# ORAL DELIVERY OF ASCORBIC ACID STABILIZED RIFAMPICIN NANOPARTICLES FOR ENHANCED BIOAVAILABILITY OF RIFAMPICIN

**Thesis submitted to**

**THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY**

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**In partial fulfillment of the requirements for the award of degree of**

**DOCTOR OF PHILOSOPHY IN PHARMACY**

**Submitted by**


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## CERTIFICATE

I hereby declare that the Ph.D thesis entitled **"Oral delivery of ascorbic acid stabilized rifampicin nanoparticles for enhanced bioavailability of rifampicin"** submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, is a record of independent work carried out in the Department of Pharmaceutics, Swamy Vivekanandha College of Pharmacy, Tiruchengode under the direct supervision and guidance of **Dr. N. N. Rajendran**, M.Pharm., Ph.D., for the partial fulfillment for the degree of **DOCTOR OF PHILOSOPHY IN PHARMACY.** This work is original and has not been submitted earlier for the award of any other degree of this or any other university.

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**(Dr.S.MOHAN)**

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# LIST OF PUBLICATIONS

1. **Subashini Rajaram**, Rajendran Natham. Design and characterization of ascorbic acid stabilized rifampicin nanoparticles for oral delivery, International Journal of Biological & Pharmaceutical Research, (Accepted; Impact factor-1.34).
2. **Subashini Rajaram**, Rajendran Natham. Influence of formulation and process variables on the formation of rifampicin nanoparticles by ionic gelation technique, Res J Pharm Biol Chem Sci, (Accepted).

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# 1. INTRODUCTION

Tuberculosis (TB) is an infection caused by M*ycobacterium tuberculosis*. It is the world's second commonest cause of death from infectious disease after HIV/AIDS. WHO declares TB as a public health emergency. The prevalence of TB is higher in south East Asia and sub Saharan Africa as compared to western countries. The prevalence of all forms of TB in India is estimated 5.05/1000 and of smear positive cases 2.27/1000[1].

Treatment of TB was initially complicated and challenging as it requires administration of drugs over a long period. This resulted in poor patient compliance. To overcome this disadvantage, a multi drug regimen was recommended that shortened the duration of treatment of TB. The anti-tuberculous drugs include rifampicin (RIF), isoniazid (INH), ethambutol (ETH), pyrazinamide (PYZ), streptomycin as first line drugs and capreomycin, anamycin, ethionamide, para-amino salicylic acid, cyclosporine, thiacetazone, ciprofloxacin, levofloxacin, ofloxacin, and sparfloxacin as second line drugs. The first line drugs are recommended for six months duration of treatment and the second line drugs for two months treatment. Initially the first line anti-TB drugs were administered separately and later on a fixed dose formulation of the first line drugs were recommended to overcome the bacterial resistant encountered with individual drugs administration.

Rifampicin is well absorbed from the stomach, whereas isoniazid is comparatively well absorbed from all three segments of the small intestine[2]. However rifampicin degrades in the stomach and the degradation has varied from 8.5-50%, during the gastric emptying time for most dosage forms in humans[3] and decomposition of rifampicin is further influenced by the presence of isoniazid in stomach after ingestion[4]. To improve this disadvantage isoniazid was administered in enteric coated form to prevent its release in the stomach so as to protect rifampicin from isoniazid induced degradation in the stomach. Despite this disadvantage the fixed dose combination (FDC) of rifampicin, isoniazid, ethambutol, pyrazinamide remains in use in the treatment of TB to attract patient compliance; However the bioavailability of rifampicin has become unacceptable in a number of FDC anti- TB formulations.

Various pharmaceutical approaches were attempted to improve the bio availability of rifampicin. Controlled drug delivery system, nanoparticles, liposomes, and microspheres were developed for the sustained drug delivery of anti-TB drugs that have demonstrated better chemotherapeutic efficacy when investigated in animal models[5]. Among these approaches nanoparticulate delivery of anti-TB drugs has assumed significance recently. Nanoparticles are stable solid colloidal particles consisting of biodegradable polymers or lipid materials and range in the size from 10 to 1000nm. Drugs can be absorbed onto the particle surface, entrapped inside the polymer/lipid or dissolved within the particle matrix[6].

Nanoparticular drug delivery though was focused initially on anti-cancer drug in the area of oncology its utility is extended to other areas of diseases including TB. It has been established in animal model that oral nanoparticle based anti-TB drug therapy can allow for reduction in dosing frequency for better management of TB. The past several years have seen the development of a number of rifampicin controlled release formulations for the improvement of clinical efficacy of the drug and patient compliance[2].

In earlier studies, Dalencon et al reported the loading of rifabutin in nanocapsules, and the preparation was evaluated in experimental toxoplasmosis[7].

Lopes et al reported the encapsulation of ethionamide, a second- line antitubercular drug[8]. Evaluation of three front-line antituberculosis drugs (RIF, isoniazid, pyrazinamide) co-encapsulated in PLGA NPs was carried out by Pandey et al[9]. Anti tuberculosis drug-loaded solid lipid NPs have produced encouraging results[10].

Physicochemical properties play a major role to generate pharmaceutically acceptable nanoparticulate system. To obtain biocompatible nanoparticle system, molecular weight of polymers, concentration of surfactants and their influence on particle size and release need to be considered. In nanoparticle system particle size becomes increasingly importance since small particle have high absorption area, therefore they penetrate cell membrane more efficiently[11]. This may depend on the polymer molecular weight, properties of drug and device characteristics (preparation conditions, particle size, morphology and drug loading) [12]. Surfactants are used to stabilize nanoparticles by hindering their growth. The increase in the surfactant amount in colloidal dispersions may contribute to the reduction of mean particle size because of the surface active properties of surfactants[13]. They improve the stability of system through static electricity repulsive forces, steric hindrance, and Van der waals force by absorbing on to the surface of nanomaterials[14,15].

Despite several approaches attempted by researchers in the past, the improved bioavailability of rifampicin either from rifampicin alone or from fixed dose formulations has not been successfully achieved owing to degradation of rifampicin in the acidic environment of the stomach.

Previous study reveals that rifampicin collected in the plasma sample can be stabilized by using ascorbic acid as anti-oxidant. It has been reported that rifampicin degrades in plasma at ambient temperature, and a 54% loss was observed within 8 hrs. This degradation can be effectively prevented by adding ascorbic acid, thus prolonging stability for up to 12 hrs[16]. Additionally administration of ascorbic acid (1000 mg/day) is recommended in tuberculous patient[17]. Besides, ascorbic acid is also added to the medium as an anti-oxidant to prevent oxidative degradation of rifampicin during the *in-vitro* diffusion[18] and dissolution[19] studies.

Different polymers have been recommended for preparation of nanoparticles. Polymers may be bio degradable or non degradable. Biodegradable polymers are preferred as they are eliminated from the body and so polymer induced toxicity can be ruled out. Natural bio degradable polymers are more desirable as they are highly biocompatible. As TB requires long term treatment, naturally occurring bio degradable polymers are recommended for chronic infections[20]. Considering this, chitosan (CS) has been chosen as a polymer for the development of nanoparticles in the study. Chitosan is a naturally occurring bio compatible cationic polysaccharide obtained from the deacetylation of chitin. Compared to other natural polymers chitosan has a positive charge and is mucoadhesive and it is used extensively in drug delivery applications[21]. Besides chitosan being natural is considered ideal polymer for development of drug delivery systems used for chronic disease. They have the capacity to protect sensitive bioactive macromolecules from enzymatic and chemical degradation during storage[22]. Chitosan has many advantages particularly for developing micro/nanoparticles. These include its ability to control the release of active agents; it avoids the use of hazardous organic solvents while fabricating particle since it is soluble in aqueous acidic solution[23]. There is a growing interest in producing nanoparticles containing antituberculous drugs using natural polymer such as chitosan[24].

Nanoparticles have been prepared most frequency by three methods (1) dispersion of performed polymers (2) polymerization of monomers and (3) ionic gelation or coacervation of hydrophilic polymers. However, other methods such as supercritical fluid technology[25] and particle replication in non wetting templates[26] have also been described in the literature for production of nanoparticles. The latter was claimed to have absolute control of particle size, shape and composition, with could set an example for the future mass production of nanoparticles in industry.

Thus, the literature reveals the importance of nanoparticulate approach for improving bio availability of rifampicin. Besides naturally occurring polymer such as chitosan is recommended for development of nanoparticles for chronic infections like TB. The usefulness of ascorbic acid in stabilizing rifampicin against degradation in acidic environment of stomach has not been established, although ascorbic acid finds its use in dissolution medium or in plasma sample as stabilizing agent. More importantly, in clinical practice, daily administration of ascorbic acid (1000mg/day) is recommended to control tuberculous by promoting immune response.

Considering the above factors, a two directional approach was attempted in the present study to investigate, first, whether ascorbic acid can protect rifampicin from degradation in the acid environment in the presence/absence of isoniazid and second, whether nanoparticulate delivery of rifampicin admixed with ascorbic acid can improve bioavailability of rifampicin.

# 2. AIM AND OBJECTIVE

The present study attempted to develop ascorbic acid stabilized rifampicin nanoparticles for oral delivery for enhanced bioavailability of rifampicin and evaluate for physicochemical, *in-vitro* release and pharmacokinetics characteristics.

**The objectives of the study were as follows**

1. Preparation of standard curve of rifampicin in 0.1N HCL
2. Preparation of rifampicin loaded chitosan nanoparticles by ionic gelation method with different variables such as
   a) Chitosan of different grades (150KDa, 300KDa, 450KDa)
   b) Tween 80 in 0.25%, 0.5%, 1.0% concentrations
   c) Stirring speed at 400rpm, 800rpm, 1200rpm
3. Preparation of rifampicin-ascorbic acid loaded chitosan nanoparticles by ionic gelation method from best formulation of rifampicin nanoparticles.
4. Evaluation of rifampicin loaded chitosan nanoparticles
   a) Drug and carrier interaction by FTIR Spectroscopy.
   b) Thermal analysis by Differential Scanning Calorimetry (DSC).
   c) Morphology by Scanning Electron Microscopy (SEM)
   d) Surface characteristics by Zeta potential analyzer
   e) Particle size determination by Zetasizer
   f) Rifampicin encapsulation efficiency and loading capacity of the nanoparticles.

g) *In-vitro* dissolution stability study of

- Rifampicin
- Rifampicin with isoniazid
- Rifampicin with ascorbic acid (125mg)

- Rifampicin with ascorbic acid (250mg)
- Rifampicin with ascorbic acid (500mg)
- Rifampicin with ascorbic acid (1000mg)
- Rifampicin with ascorbic acid (125mg) and isoniazid
- Rifampicin with ascorbic acid (250mg) and isoniazid
- Rifampicin with ascorbic acid (500mg) and isoniazid
- Rifampicin with ascorbic acid (1000mg) and isoniazid

h) *In-vitro* diffusion, *in-vivo* pharmacokinetic study of

- Control (Rifampicin)
- Rifampicin+ Isoniazid
- Rifampicin-ascorbic acid
- Rifampicin-ascorbic acid + Isoniazid
- Rifampicin nanoparticles
- Rifampicin nanoparticles + Isoniazid
- Rifampicin-ascorbic acid nanoparticles
- Rifampicin-ascorbic acid nanoparticles + Isoniazid

# 3. REVIEW OF LITERATURE

## 3.1 Epidemiology of tuberculosis

TB is an infection caused by *M. Tuberculosis.* The highest prevalence of tuberculosis infection and estimated annual risk of tuberculosis infection are in sub-Saharan Africa and Southeast Asia. It is endemic in most developing countries and resurgent in developed and developing countries with high rates of human immunodeficiency virus (HIV) infection, particularly in Africa, although increases are also expected in Southeast Asia. In many industrialized countries, TB has recently failed to decline, and in Eastern Europe and the former Soviet Union, cases and deaths are increasing. Drug resistance is a serious problem, especially in the United States[27].

India is classified along with the sub-Saharan African countries to be among those with a high burden and the least prospects of a favorable time trend of the disease as of now (Group IV countries). The average prevalence of all forms of tuberculosis in India is estimated to be 5.05 per thousand, prevalence of smear-positive cases 2.27 per thousand and average annual incidence of smear-positive cases at 84 per 1,00,000 annually[1].

TB remains a major cause of morbidity and mortality worldwide in the 21$^{st}$ Century. The WHO and other organizations have put vast resources into studying the disease, as well as implementing and monitoring treatment. There are large disparities between the rates of TB in children in resource poor countries and those in industrialized countries. Factors such as poverty, overcrowding and HIV infection have contributed greatly to the resurgence of childhood TB, particularly in Sub-Saharan Africa. The mortality rates from TB in children from resource-poor counties are unacceptably high. While there are many challenges in the diagnosis and treatment of TB in children, perhaps the greatest challenge globally is to begin to identify the extent of disease in this forgotten group[28]. The TB incidence (number of new cases arising each year) and mortality in each of the WHO regions is depicted in Figure 1[29].

Effective control of tuberculosis (TB) requires an understanding of the changing epidemiology of the disease. The success in reducing the tuberculosis burden reflects several factors, including improved public health efforts, physician and patient education, infection control measures, and the use of directly observed therapy (DOT). Future efforts to curtail the incidence of TB will require vigilant public health efforts, improving education of patients and health care personnel, identifying mechanisms and routes of transmission, and assuring adequate treatment and prophylactic regimens among infected individuals[30].

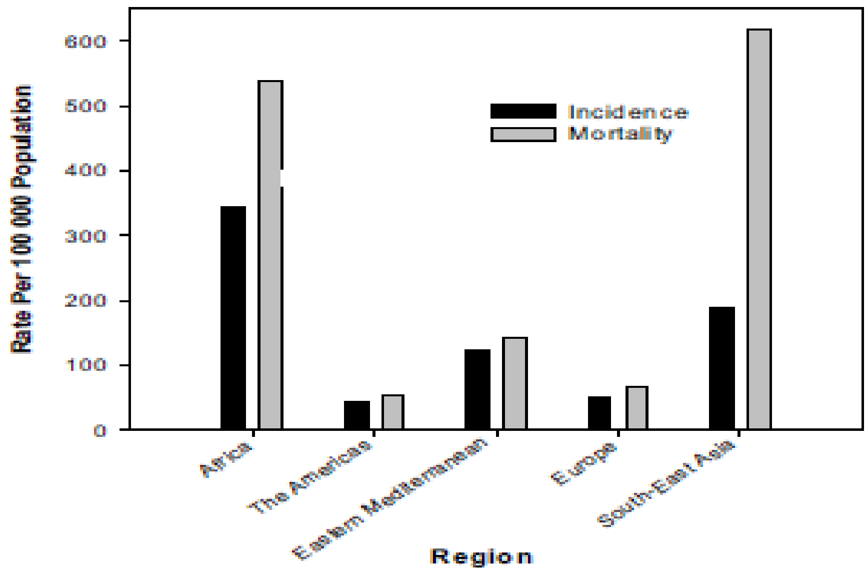


**Figure 1. Estimated TB incidence and mortality in 2003.**

**Data extracted from WHO Tuberculosis data sheet (WHO-Geneva 2003).**

## 3.2 Current chemotherapy for tuberculosis

Since the control measures for TB such as Bacillus Calmette-Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, treatment with anti-tubercular (anti-TB) drugs becomes the only option available. The goals of treatment are to ensure cure without relapse, to prevent death, to impede transmission, and to prevent the emergence of drug resistance. Long-term treatment with a combination of drugs is required[31]. Treatment of active TB with a single drug should never be attempted, and a single drug should never be added to a failing regimen, the result being development of MDR TB[32]. As suggested by WHO[33], treatment of TB and drug resistant cases requires multi-drug therapy, comprising:

1. An initial intensive phase of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ), and ethambutol (ETB) daily for 2 months.
2. A continuation phase of RIF and INH for a further 4 months, either daily or 3 times per week, to be administered as advised in Table 1.

**Table 1 Regimen 1 for treatment of new smear positive adult patients**

| | | |
|---|---|---|
| Intensive phase- 2 months<br>RIF/INH/PYZ/ETB<br>Combination tablets 120/60/300/200mg daily 5 days per week | Under 50 kg<br><br>4 tablets | Over 50 kg<br><br>5 tablets |
| Continuous phase<br>RIF/INH<br>Combination tablets 150/100mg<br>Combination tablets 150/300 mg | Under 50 kg<br><br>3 tablets<br>- | Over 50 kg<br><br>-<br>tablets |

Data extracted from (Gibbon C)[34].

INH eradicates most of the rapidly replicating bacilli in the first 2 weeks of treatment, together with streptomycin and ETB. Thereafter, RIF and PYZ have an important role in the sterilization of lesions by eradicating organisms; these two drugs are crucial for successful 6-months treatment regimens. RIF kills low or non-replicating organisms and the high sterilizing effect of PYZ serves to act on semi dormant bacilli not affected by any other anti-TB agents in sites hostile to the penetration and action of the other drugs[35, 36].

INH and RIF, the two most potent anti-TB drugs, kill more than 99% of tubercular bacilli within 2 months of initiation of therapy[37,38]. Using these drugs in conjunction with each other reduces anti-TB therapy from 18 months to 6 months. The sites of action of these principal anti-TB agents are schematically illustrated in Figure 2[39,40,41].

The emergence of strains resistant to either of these drugs causes major concern, as treatment is then deferred to drugs that are less effective, have more toxic side effects, and result in higher death rates, especially among HIV-infected person[41]. The current armamentarium of drugs available for the treatment of TB, their mechanism of action, and activity have been reviewed by numerous authors[2] and appear in Table 2.

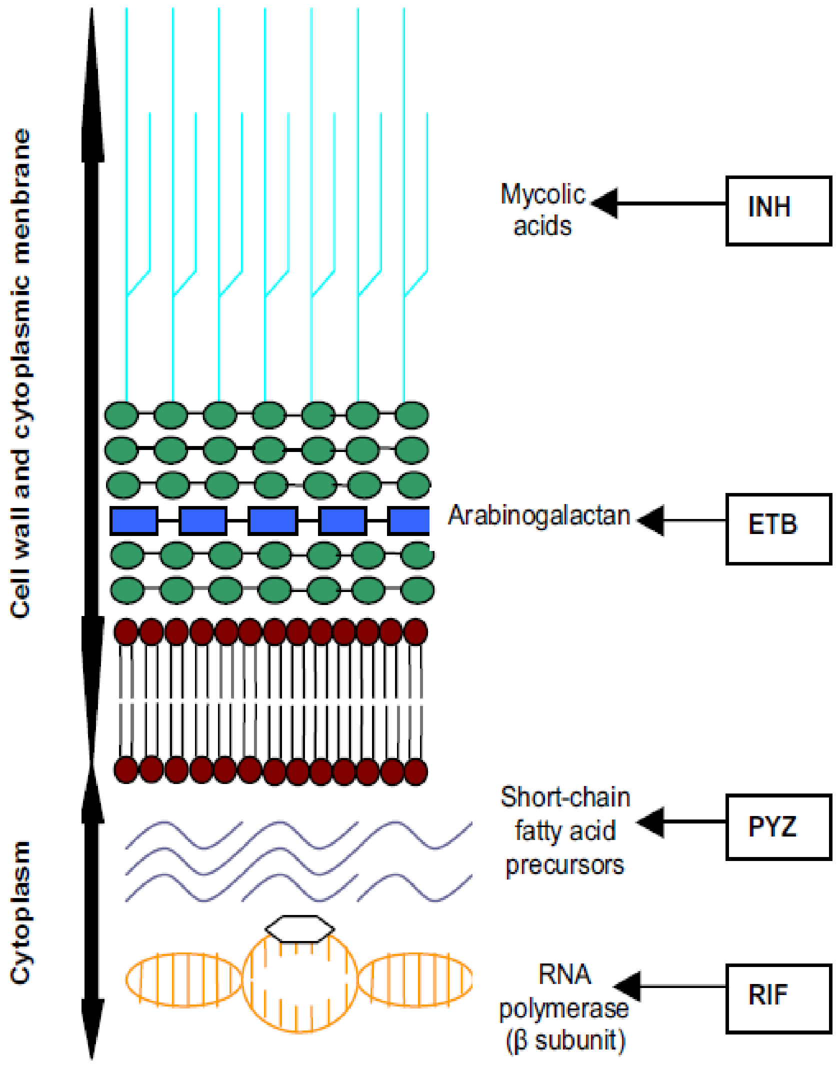


**Figure 2. Sites of action of the principle anti-TB drugs**

**Table 2 Classes of anti-TB drugs**

| Agent | Mechanism of action | Activity against *M. tuberculosis* |
|---|---|---|
| **First-line agents** | | |
| Rifampicin (RIF) | Inhibits bacterial RNA synthesis by binding to the β subunit of bacterial DNA-dependent RNA-polymerase (DDRP), inhibition of DDRP leads to blocking of the initiation chain formation in RNA synthesis. One of the most effective antituberculosis agents available and is bactericial for intra- and extra-cellular bacteria. | RIF inhibits susceptible organisms at concentrations of less than 1 μg/ml. |
| Isoniazid (INH) | Most active drug for the treatment of TB caused by susceptible strains. Is a pro-drug activated by KatG, which exerts its lethal effects through inhibition of synthesis of mycolic acids, an essential component of mycobacterial cell walls, through formation of a covalent complex with an acyl carrier protein (AcpM) and KasA, a beta-Ktoacyl carrier protein synthetase. | INH inhibits tubercle bacilli as a concentration of 0.2 μg/ml. |
| Pyrazinamide (PYZ) | Converted to the active pyrazanoic acid (encoded by pncA) by pyrazinmidase in susceptible organisms. Pyrazanoic acid lowers pH in the immediate surroundings of *M.tuberculosis* | Inhibits *M.tuberculosis* and other mycobacteria at concentrations of 20μg/ml. |

| | | |
|---|---|---|
| | – organisms is unable to grow. May also function as an antimetabolite of nicotinamide and interfere with the synthesis of NAD, inhibiting the synthesis of short-chain, fatty-acid precursors. | |
| **Aminoglycosides** (injectable)<br>Streptomycin, kanamycin, amikacin, capreomycin. | The aminoglycosides are irreversible inhibitors of protein synthesis through binding to specific 30s-submit ribosomal proteins. | Bacterial in vitro and in vivo clinical data support use. |
| | Inhibit bacterial DNA synthesis through inhibition of bacterial topoisomerase II (DNA gyrase) and topoisomerase IV, which are responsible for the relaxation of supercoiled DNA and the separation of replicated chromosomal DNA, respectively | Bactericidal broad spectrum antibacterials in-vitro and in-vivo clinical data support use. Ciprofloxacin and levofloxacin inhibit strains of *M. tuberculosis* at concentrations of less than 2µg/ml. Newer agents (moxifloxacin, gatifloxacin, sparfloxacin have lower minimum inhibitory concentrations. |

| | | |
|---|---|---|
| **Other drugs** | | |
| Clofazimine | Potentially useful agents with conflicting animal or clinical evidence or agents with unclear efficacy because of possible cross-resistance.<br>Unknown but may involve DNA binding possesses direct anti mycobacterial and immunosuppressive properties | Bacteriostatic *in-vitro* MIC 90<1.0mg in-vitro. Apt concentrations attainable in vivo particularly in macrophages<br>β-lactams in combination with beta lactamase inhibitors bactericidal in vitro. |
| Amoxicillin/ clavulanic – acid | Amoxicillin (a penicillin) inhibits cell wall synthesis, Clavulinic acid is a beta-lactamase inhibitor | Although in-vitro anti-myco bacterial properties reported data from animal in vivo studies conflicting. |
| Clarithromycin | Inhibition of protein synthesis via binding to 50S ribosomal RNA as aminocacyl translocation reaction and the formation of initiation complexes are blocked | May be useful against some isolates of MDRTB (resistant to RIF in vitro but sensitive to rifabutin). Effective in prevention and treatment of disseminated atypical mycobacterial infection in AIDS patients with CD4 counts < 50. |
| Rifabutin | Activity is similar to that of rifampicin, inhibits bacterial RNA synthesis by binding strongly to the β submit of bacterial DNA dependent RNA-polymerase | *In vivo* and *in vitro* evidence of bacteriostatic activity. Cross – resistance frequently seen between thiacetazone and both INH and ethionamide. |

TB is treated with a multi-drug regimen, and is thus exceptionally vulnerable to incidences of side effects, unsatisfactory patient compliance and slow improvement of patients[42]. Therefore, despite the availability of these highly effective treatments for TB, cure rates remain low as commercial anti-TB formulations are inconvenient to administer and patients do not take the prescribed medications with sufficient regularity and duration to achieve a cure[43].

Patients have to consume a large number of tablets (up to eight at one time), which is a common cause for non-compliance. It can be anticipated that non-optimal application of these short course regimens will result in the deterioration of their therapeutic potential, an escalation in the mortality rate and increased risk of developing acquired drug resistance[29,44,45]. Resistance of *M.tuberculosis* to anti-TB agents is a worldwide problem in both immune competent and HIV-infected population[46,47].

### 3.3 Novel drug delivery systems for the treatment of TB

Chemotherapy of TB is complicated by the need of multidrug regimens that need to be administered over long periods. Poor patient compliance is the single most common reason for chemotherapy failure in TB[42]. To minimize toxicity and improve patient's compliance, extensive progressive efforts have been made to develop various implant, micro particulate, and various other carrier-based drug delivery systems to either target the site of *M. tuberculosis* infection or reduce the dosing frequency, which forms an important therapeutic strategy to improve patient outcomes[5,48]. The systems under discussion employ either biodegradable polymers or systems requiring removal after use, and can release the drug either by membrane or matrix-controlled diffusion.

Recent trends in controlled drug delivery have seen micro encapsulation of pharmaceutical substances in biodegradable polymers as an emerging technology. Carrier or delivery systems such as liposomes and microspheres have been developed for the sustained delivery of anti-TB drugs and have demonstrated better chemotherapeutic efficacy when investigated in animal models (e.g. mice)[5]. Anti-TB drugs have been successfully entrapped and delivered in biodegradable polymers such as poly (DL-lactide-co-glycoside) (PLG), which are biocompatible and release drug in a controlled manner at therapeutic levels[49].

Dutt and Khuller (2001)[50] have entrapped INH and RIF in PLG polymers. When injected subcutaneously as a single dose, the micro particles, having a diameter ranging from 11.75 μm to 71.95 μm, provided sustained release of drugs over 6–7 weeks when tested in mice. The authors previously observed that particles with a size range >10 μm remained at the site of injection forming a depot. The entrapped contents of the micro particles were gradually released by diffusion through the polymeric particles. Such depots can show release profiles extending over several months culminating in degradation of the entire polymeric device. However, these formulations have to be injected either subcutaneously or intravenously, and the pain and discomfort associated with these routes of administration, in general, is often not acceptable. Hence, there is a continuous need to develop an oral drug delivery system that is convenient for patients[18].

Amidst these concerns, Ain *et al* (2002)[51] reported the pharmacokinetics of PLG encapsulated anti-TB drugs; orally administered either individually or in combination in mice. A study conducted by Pandey *et al* (2003) [48] reported the formulation of three frontline anti-TB drugs, i.e. RIF, INH and PYZ encapsulated in PLG nanoparticles. On oral administration of drug-loaded nanoparticles to *M.*

*tuberculosis*-infected mice at every 10th day, no tubercle bacilli could be detected in the tissues after 5 oral doses of treatment. Therefore, oral nanoparticle-based anti-TB drug therapy can allow for a reduction in dosing frequency for better management of TB.

Prabakaran[42] developed an osmotically regulated capsular multi-drug oral delivery system comprising asymmetric membrane coating- and dense semi permeable membrane coating-capsular systems for the simultaneous controlled administration of RIF and INH for the treatment of TB. This was in an attempt to reduce the problems associated with multidrug therapy. The modified asymmetric system provided satisfactory sustained release of RIF and INH, with an initial burst release that may be sufficient to achieve minimum effective concentration in blood. Thereafter, the system provided the release of the drugs in a near zero order rate – an ideal release profile for controlled drug delivery. In turn, this would improve the safety profile of the drugs and enhance the activity duration of drug exhibiting short half-lives. The once daily system is optimal, and could potentially enhance patient compliance.

Further attempts to solve the problems inherent in multidrug therapy have included the development of biodegradable polymeric micro- or nanoparticulate carrier systems to target alveolar macrophages that harbour *M.tuberculosis*. In the case of pulmonary TB, delivering the drug directly to the site of infection through inhalation of an aerosolised delivery system has the inherent advantages of bypassing first-pass metabolism and maintaining local therapeutically effective concentrations with decreased systemic side effects[15]. Because *M. tuberculosis* is known to infect alveolar macrophages and affect the pathogenesis of TB, there have been renewed interests in targeting of anti-TB drugs to these cells. Despite the success of these systems in targeting and providing sustained release of anti-TB drugs to alveolar macrophages, the methods used to generate particles in these studies vary in their capability for the production of reproducible particles with the optimal size for inhalation therapy (i.e. <5µm).

Barrow et al (1998)[52] formulated RIF-loaded microspheres using the method of solvent evaporation, aiming to maintain a size of 1 to 10-µm. Only the size distributions of two formulations were reported, being 3 to 4 µm and distribution demonstrated a Gaussian curve.

Dutt and Khuller ( 2001)[53] encapsulated INH and RIF into hardened PLG micro particles by a double emulsification solvent evaporation procedure, and these had a resultant volume mean diameter of 11.75 µm for INH microparticles and 11.64 µm for RIF microparticles. These are currently undergoing Phase I trials. Sharma et al (2001)[54] incorporated both INH and RIF into PLG microspheres using a combination of solvent extraction and evaporation, but these particles had a mean diameter of 6.214 µm and only 38% of the microspheres fell in the size range of 0.5–3 µm. Suarezet et al (2001)[55] attained the airway delivery of RIF microparticle shaving volume median diameters of 2.76 ± 1.57µm. O'Hara and Hickey[56] succeeded in obtaining RIF loaded PLG particles with median diameters by volume of 2.76 µm and 3.45 µm by spray drying and solvent evaporation respectively.

Zhou et al (2005)[15] did achieve the formulation of spherical micro particles between 1 and 3 µm in diameter. The microparticles, prepared by the precipitation with a compressed anti solvent process, were evaluated for their potential in targeting an ionizable prodrug of INH, isoniazid methane sulfonate (INHMS), for sustained delivery of INH to alveolar macrophages.

Most recently Zahoor et al (2006)[57] undertook pharmacokinetic and chemotherapeutic studies with aerosolized alginate nanoparticles encapsulating INH, RIF and PZA and RIF, INH, PYZ, ETB. The nanoparticles were prepared by cation-induced gelification of alginate and were 235.5± 0 nm in size, with drug encapsulation efficiencies of 70–90% for INH and PZA and 80–90% for RIF and 88–95% for EMB. The majority of particles (80.5%) were in the respirable range, with a mass median aerodynamic diameter of 1.1 ± 0.4 μm and geometric standard deviation of1.71 ± 0.1 μm. The chemotherapeutic efficacy of three doses of drug-loaded alginate nanoparticles nebulised 15days apart was comparable with 45 daily doses of oral free drugs. Thus, inhalable alginate nanoparticles could potentially serve as an ideal carrier for the controlled release of anti-TB drugs. Clinical trials are envisaged in the future for evaluation of this system before use in humans.

Pandey and Khuller[58] evaluated the chemotherapeutic potential of nebulised solid lipid nanoparticles (SLNs) incorporating RIF, INH and PYZ against experimental TB. SLNs are nanocrystalline suspensions in water, prepared from lipids, which are solid at room temperature. The SLNs, prepared by the emulsion solvent diffusion technique, possessed a favourable mass median aerodynamic diameter suitable for broncho alveolar drug delivery. Following a single nebulisation to guinea pigs, therapeutic drug concentrations were maintained in the plasma for 5 days and in the organs for 7 days whereas free drugs were cleared after 1–2 days.

Vyas[59] formulated aerosolized liposomes incorporating RIF via a cast-film method employing egg phosphatidylcholine- and cholesterol based liposomes. Liposomes coated with alveolar macrophage-specific ligands demonstrated preferential accumulation in alveolar macrophages, maintaining high concentrations of RIF in the lungs even after 24 hours.

In another approach to solve the predicament of poor patient compliance, depot-delivery of anti-TB drugs has been investigated. Studies have demonstrated that a single implant of INH in polylactic-co-glycolic acid (PLGA) copolymer could ensure sustained levels of free INH for a period of up to 8 weeks following implantation in rabbits[49].

A number of the aforementioned developments in drug delivery represent attractive options with significant merit, and the pertinent points regarding each exemplary investigation are summarized in Table 3. However, the need to develop an oral drug delivery system with improved patient acceptance is affirmed by the accelerated pace of oral drug delivery system development fostered by the need to deliver medications to patients more efficiently and with fewer side effects, especially in developing countries where controlled-delivery implants and injectables could be too expensive.

**Table 3 Synopsis of novel anti-TB drug delivery systems**[2]

| Drug | Delivery System and Polymer employed | ROA | Preparation Method | Characterization studies and system suitability |
|---|---|---|---|---|
| INH | Porous, non-porous and hardened microparticles employing PLG | SC injection | Double emulsification solvent evaporation | **Size*:*** Mean volume diameters were 62.11 µm, 71.95 µm, 11.75 µm, for porous, non-porous and hardened micro particles, respectively.<br>**In vitro studies**: sustained release of INH up to 6 days from non-porous microparticles. Porous microparticles released INH over 3 days.Hardened PLG microparticles sustained releases of INH for up to 7 weeks.<br>**In vivo disposition studies (In mice):**_porous and non-porous microparticles released INH in plasma for up to 2 days. Hardened PLG microparticles sustained release INH for up to 7 weeks. Concentrations of INH obtained were higher than the MIC of INH. |

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| RIF, INH, PYZ, ETB | Microparticles employing PLG | Oral, singly or in combination | Double emulsification solvent evaporation | **DEE**: 8-10% for PZA: 10-11% for INH and 12-18% for RIF. **Size**: diameters were I. II µm for I.40 µm for RIF and 2.20 µm for PZA microparticles. **In vitro studies**: entrapped drugs were released in a sustained manner. In the intestinal fluid drug release was obtained for up to 20 days. **In vivo studies:** entrapped drugs remained in circulation up to 72 has compared to free drugs (eliminated within 24h). Level of PLG encapsulated INH was found to be higher than its MIC value (0.1 µm/ml). **Pharmacokinetics**: (PLG encapsulated of drugs and free drugs): increased C, AUC and $t_{1/2}$ (e) when drug were given entrapped in PLG microparticles indicated the potential of PLG for effective treatment TB. |

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| RIF, INH, PYZ | Nanoparticles employing PLG | Oral | Multiple emulsion technique | **Size**: majority (>80%) in the size range of 186-290nm, polydispersity index of 0.38=0.04<br>**DEE**: 56.9±2.7% for RIF, 66.3=508% for INH and 68±5.6% for PZA<br>**Drug loading**: 570 to 680 mg drug per gram of polymer.<br>**In vitro studies:** drug release profile in PBS showed an initial (up to 48h) burst release followed by a negligible release of either drug up to 6 weeks.<br>**In vivo studies**: (experimental infection and chemotherapy): following oral administration of drug loaded nanoparticles to M. tuberculosis infected mice at every 10$^{th}$ day-no tubercle bacilli could be detected in the tissues after 5 oral doses of treatment. |

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| RIF, INH | Osmotically regulated capsular multi-drug oral delivery system employing HPMC and NaCMC | Oral | Phase inversion process-precipitation of membrane structure on stainless steel mould pin | **SEM**: Porous structure of the membranes was evident. **In vitro studies**: sustained release of RIF and INH with Intel burst release. Which may be sufficient to achieve MIC in blood. Therefore the system sustained the release of the drugs in a near zero order rate. **In vitro release kinetics:** first order kinetics. Statistical analysis of release rate data-modified asymmetric system the preferred system. |
| INH, RIF | Microparticles employing PLG | SC, inhaled | Double emulsification solvent evaporation | **Size**: volume mean diameters of 11.75 µm (INH – loaded) and 11.64 µm (RIF-loaded) **DEE**: 10-11% (INH-loaded) and 12-14% (RIF-loaded) **In vivo-combination drug disposition studies and experimental infection and chemotherapy studies:** Single dose of PLG microparticles-sustained release of INH and RIF for up to 7 and 6 weeks, respectively. Free drugs in combination injected in the same doses were detectable in vivo up |

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| | | | | to 24 h only. One dose of PLG microparticles cleared bacteria more effectively from lungs and liven in experimental marine model of TB (compared with a daily administration of the free drugs) phase trials. |
| INH, RIF, PZA and RIF, INH, PYZ, ETB | Nanoparticles employing alginate | Inhaled | Cation- induced gelification of alginate | **Size**: 235.5 ±0 nm in size, with majority of particles (80.5%) were in the respirable range, with mass median aerodynamic diameter of i.± 0.4µm and geometric standard deviation of 1.71±0.1µm. **DEE**: 70-90% for-90% for INH and RIF, 80-90% for RIF and 88-95% for ETB. **In vivo studies (disposition studies and chemotherapeutic studies):** The formulation was orally administered to mice at two does levels. A comparision was made in mice receiving free drugs at equivalent doses. Relative bioavaibilities of drugs encapsulated in alginate nanoparticles significantly higher compared with oral free drugs. Drug levels were maintained ar or above the MIC90 post nebulisation until Day 15 in organs (lungs, liver and spleen) |

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| | | | | after administration of encapsulated drugs, whilst free drugs stay at or above the MIC90 up to Day 1only irrespective of dose. Clinical trials envisaged in the future. |
| RIF , INH and PYZ | Nebuilised SLNs Prepared from nanocryalline lipid suspension in water | Inhaled | Emulsion solvent Diffusion Technique | **Size:** Favourable mass median aerodynamic diameter suitable for bronchoalveolar drug delivery<br>**In vivo Studies:** Theraputic experimental TB drug concentrations were maintained in the plasma for 5 days and in the the organs for 7 days whreas free drugs were cleared by 1-2 days<br>**Modification:** Imparted negative charge (DCP) or by coating them with alveolar macrophage specify ligands (MBSA and 0-SAP). |
| RIF | Aerosoliposme formulated using Egg PC- and Chol-based Liposomes | Inhaled | Neutral Liposomes were prepared by case film method | **Size:** Netural and negatively charged liposomes composed of PC:Chol:DCP had average vesicle size of 2.32±0.48 µm, respectively. MBSA-Coated liposomes size: 3.64±0.65µm, 0-SAP-coated vesicles size: 3.85±059µm.<br>**DEE**: 47.4±2.7%. |

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| | | | | **In vivo studies:** Precent viability of mycobocterum smegnatis inside macrophages (invitro) after administration od drug (in vivo) was 7-11% (ligand-anchoraed liposomal aerosols), 45.7 and 31.6% case of plain Drug and plain neutral liposomal aerosol(based on PC: Chol) – treated macrophages. Preferential accumulation of MBSA-and 0-Sap- coated formulations in alveolar macrophages. Drug was estimated in the lung in high concentration (even after 24 h). |
| INH | Implementaion from PLGA | Depot | PLGA polymer rods | **In vivo studies**: Rods implanted in the back of rabbits under anaesthesia. Concentations of INH and acetyl isoniazid in serum and urine determined by HPLC. Concentrations of INH$\geq$ 0.2 µg/ml were found both in serum and urine up to 63 days after implant. Urine specimen obtained at 6 weeks after implant inhibited the growth of M. tuberculosis in vitro measured by radiometric (Bactec) Method. |

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|---|---|---|---|---|
| INH, PYZ | Single implants prepared from PLGA | Depot | Depot drug preparation | **In vivo Studies** : 3 times the daily dose of PYZ contained in single PLGA polymer implant – no burst levels of the drug evident after administration – sustained levels up to 54 days. Chemotherapeutic activity Investigated in mice of the single PLGA Polymer Implants similar to standard oral treatment with the two drugs given daily for 8 weeks, determined by mortality and CFU counts of tubercle bacilli from lungs and spleen. |

Chol = Cholesterol, DCP = Dicetylphosphate, DEE= Drug entrapment efficiency, HPLC= High performance liquid chromatography, HPMC = Hydroxy propyl methyl cellulose,MIC -Minimum inhibitory concentration, NaCMC = Sodium carboxy methyl cellulose, PBS= Phosphate-buffered saline, PC= Phosphatidylcholine, MBSA = Methylated bovine serum albumin, and O-SAP=O-sterile amylopectin, ROA= Route of administration, SC=Subcutaneous, SLN=Solid lipid nanoparticles.

### 3.4 Causative factors for poor bioavailability of rifampicin

#### 3.4.1 Drug adsorption by the excipients

When rifampicin is administered along with *p*-amino salicylic acid, which is also an anti-tubercular drug, its absorption is delayed and the *C*max and the AUC values are reduced to a half, than that obtained by the administration of rifampicin alone[60]. This observation has been attributed to the adsorption of rifampicin on bentonite, which was used as an excipient in the manufacture of *p*-amino salicylic acid granules[61]. The bioavailability of rifampicin is also reported to reduce significantly when it is administered along with antacids[62]. The effect of antacids on the bioavailability of rifampicin is shown to be in the order of magnesium trisilicate > aluminium hydroxide > sodium bicarbonate, and is ascribed to the combined effects of gastric pH elevation, chelation of drug by aluminium ions and binding of rifampicin with magnesium trisilicate. The drug is also bound to an extent of 16–20% by the partially neutralized magnesium silicate at pH 5.

It is unlikely that drug adsorption is the cause, in particular, for poor bioavailability of rifampicin from FDC products. First of all, the FDC formulations (tablets or capsules) contain excipients in amounts much lower than drugs present. Secondly, the solid formulations generally do not contain adsorbents as excipients in significant quantities. The only likelihood in that situation could be the inadvertent administration of antacids to patients along with the FDC products. But as this would happen even in formulations containing rifampicin alone, this reason even does not explain the typical poor bioavailability of rifampicin from FDC products.

### 3.4.2 Formulation factors

The formulation factors certainly can be a cause of variable bioavailability, as the performance of the formulation per se depends upon the quality of active ingredient(s), quality and combination of excipients, the process used in the manufacture, and the packaging. During development of formulations, several trials with different excipients and/or process are made, before the formulation of desired characteristics and stability is obtained. It means that intermediate formulations exist that do not conform to desired specifications. In case the person involved in formulation is not fully experienced and trained, and is not in knowledge of the intricacies of the formulation development, the end product might be a half-baked poor-performing formulation, which might get into the market.

Many pharmaceutical companies in several parts of the world do not have quality control measures available with them, and the performance of the formulations so produced cannot be judged before release. This problem is compounded by the absence of a simple in vitro test that can act as an effective substitute to in vivo bioavailability evaluation, which is a costly proposition. In such situations, formulation-to-formulation and batch-to-batch variations are bound to happen. The variations have been observed practically, for example, bioavailability of nine different single-drug rifampicin formulations of three pharmaceutical forms, syrups (2), tablets (4) and capsules (3), was evaluated by Mannisto[63] (1976) and both inter- and intra-formulation differences were observed.

Similarly, in a recent study, variations were reported in bioavailability of multiple marketed FDC products[64]. Evidently, the variations due to formulation factors occur both in single rifampicin and the FDC products, suggesting that there is a still more specific reason that is responsible for the typical overall poor bioavailability of rifampicin from FDC products, as compared with the formulations containing rifampicin alone.

### 3.4.3 Drug decomposition

The decomposition of RIF has varied from 8.5 to 50% in the acidic environment of the stomach in the time range corresponding to the gastric residence time for most dosage forms in humans (≈15 minutes to 105 ± 45 minutes)[65,3]. However, the gastric-emptying time for some single-unit dosage forms may reach 6 hours. The use of substandard FDC will ultimately result in drug resistant TB and treatment failure[43]. The factors proposed for this variation in the bioavailability of RIF from different FDC formulations include the particle size and crystalline form of the drug, manufacturing process and the excipients employed[66]. The effect of these factors, however, has not been convincingly explained in previous studies.

RIF is known to undergo hydrolysis in acidic medium to the insoluble 3-formyl rifamycin SV (3FRSV). INH accelerates degradation of RIF into this poorly absorbed derivative (3 FRSV) in the acidic environment of the stomach via reversible formation of the isonicotinyl hydrazones of 3-FRSV with INH[4]. Shishoo et al (2001)[3] has indicated that RIF in the presence of INH as a FDC may undergo greater decomposition in the acidic conditions of the stomach, as compared to when RIF is administered (orally) alone. Thus, less RIF will be available for absorption from FDCs as compared to RIF administered as a separate formulation. This will be reflected in the poor bioavailability from the former formulation.

There is thus an urgent need to modify or segregate the FDC formulation in such a way that RIF and INH are not released simultaneously in the stomach. Alternatively both drugs need to be administered separately after an interval corresponding to average gastric residence time, which is somewhat unpredictable due to high intra- and inter-subject variability[65,3,67].

Fairly recently, Chen proposed a mechanism for this apparent degradation of RIF. 3-FRSV and INH could possibly undergo Schiff's reaction to form a complex. The carbonyl groups and amine groups may rearrange to yield an ammonium ion. The C-4 hydroxyl group enhances the complex formation by possibly forming a hydrogen bond with the hydrogen atom attached to the nitrogen. This is a basic requirement of the Schiff's reaction. In addition, carboxylic acids and alcohols can also undergo carbonyl condensation reactions. INH could react with RIF in this manner, which could account for the instability of RIF when present together with INH .This interaction could also occur between RIF and PYZ, however, it has frequently been observed that INH caused further RIF stability reduction compared to PYZ. The reason for this could be due to the fact that the carboxylic acids and alcohol further undergo Fischer's esterification[68]. The hydroxyl groups of RIF are readily able to react with the aqueous carboxylic acid degradants yielded by INH and PYZ to form an ester. However, as PYZ lacks the electron-withdrawing group, such as the secondary nitrogen found on the hydrazine group of INH, there are fewer tendencies for this reaction to be expected between PYZ and RIF.

Permeability studies have demonstrated that RIF is well absorbed from the stomach due to its solubility, which has been shown to be maximal between pH 1–2. INH, although demonstrating solubility in the gastric environment, is comparatively well absorbed from all three segments of the small intestine. RIF and INH thus exhibit regional specific permeability, and the bioavailability problems associated with RIF could be overcome by developing an FDC in which the delivery of the two drugs is segregated, with RIF released in the stomach and INH in the small intestine[69]. A FDC multi particulate oral system, which boasts ease of manufacture and directly attacks RIF bioavailability concerns and poor patient compliance with existing FDC anti-TB formulations, is yet to be globally developed.

**3.5 Methods adopted to minimize/prevent the degradation of rifampicin**

Rifampicin is a first line antituberculous drug as oral dosage form. It is primarily absorbed from the stomach due to its solubility in the gastric pH, however undergoes degradation in the gastric environment and the degradation is further influenced by concurrently administered isoniazid. So it remains a concern for the bioavailability of rifampicin from a number of FDC anti TB formulations.

Rifampicin is well absorbed from the stomach due to its solubility, which is maximum between pH 1-2. Isoniazid is poorly absorbed from the stomach, but is well absorbed from all three segments of the intestine. In combination, rifampicin disappearance was enhanced in the presence of isoniazid in the stomach and jejunum, but isoniazid disappearance was not influenced by rifampicin. The study shows higher in situ rifampicin disappearance in the presence of isoniazid, attributable to drug degradation due to catalysis by isoniazid. As the two drugs show regional specific permeability, FDCs without reduced rifampicin bioavailability resulting from its decomposition in the presence of isoniazid can be designed by segregating delivery of the two drugs by around 3-4hrs. Rifampicin should be released in the stomach and isoniazid in the intestine[69].

The problem with rifampicin is its poor/variable bioavailability of rifampicin in anti-tubercular fixed-dose combination (FDC) products. They determined that rifampicin decomposition is enhanced by the presence of isoniazid in stomach after ingestion. 80–90% of rifampicin was dissolved in 0.1 N HCL within 10 min, and all samples showed an overlapping dissolution profile[4]. Degradation of rifampicin in 0.1N HCl, and simulated gastric fluid (SGF) at 37°C in 45 min (USP dissolution test conditions) in the absence and presence of isoniazid has been documented. Rifampicin alone decomposes in the described conditions to an average extent of 6.33%, while the loss of rifampicin in the presence of isoniazid increases on an average to 16.32%[3,70].

The stability of rifampicin in plasma kept at an ambient temperature for 24hrs or stored at -20ºc for 2 weeks, the possible protective effect of adding ascorbic acid is possible. Rifampicin degrades rapidly in plasma at ambient temperature, and 54% loss was observed within 8hrs. This degradation can be effectively prevented by adding ascorbic acid, thus prolonging the stability for upto 12hrs. Rifampicin occurs after storage for 1 week at $-20^0$c. However samples supplemented with ascorbic acid before freezing, no degradation was observed within 14 days[16].

Rifampicin oxidizes in solution to form rifampicin quinone. Ascorbic acid is often added to solutions of RIF to slow down this oxidation and explained on short term stability studies in plasma, no degradation was observed in thawed samples up to 9 hrs. The response after 9hr were 93.7% and 96.1% of the response at t=0hr at RIF concentrations. Degradation was observed at lower concentration of the drug

(0.5µg/ml) in the freeze thaw samples. However, at high concentration (20 µg/ml), this degradation was less evident[71].

Contact between rifampicin and isoniazid can decrease the degradation of rifampicin. Delay of rifampicin release in the acidic medium was achieved by preparing the Rifampicin-Sodium lauryl sulphate mixture in the ratio of 1:1 by co-grinding method which is a relatively simple and effective method. Thus this approach is beneficial for the segregation of release pattern of rifampicin in alkaline environment and isoniazid in the acidic environment of the GI tract, which will lead to prevent the degradation of rifampicin alone and its interaction with isoniazid[72].

### 3.6 The rationale for the development of a novel fixed dose combination anti-TB drug delivery System

Drug delivery, which takes into consideration the carrier, the route and the target, has evolved into a strategy of processes or devices designed to enhance the efficacy of therapeutic agents through modified or controlled release. This may involve enhanced bioavailability, improved therapeutic index, or improved patient acceptance or compliance. Drug delivery has been defined by Flynn as 'the use of whatever means possible, be it chemical, physicochemical or mechanical, to regulate a drug's access rate to the body's central compartment, or in some cases, directly to the involved tissues. The underlying principle that drug delivery technology can bring both therapeutic and commercial value to health care products has been widely accepted. This has created an intense need for presenting 'old' drugs, such as those encompassed in the anti-TB regimen, in new forms utilizing novel modes of delivery and dosage forms[73]. Patient failure to take the prescribed medications at the required intervals results in significant morbidity and mortality. The need for research into an oral anti-TB drug delivery system is thus warranted as the efficacy of the current regimen may be improved if the delivery rate, biodegradation, and site-specific targeting can be predicted, monitored and controlled.

From a financial and a global health care perspective, finding new ways to administer the anti-TB drugs in oral form and delivering the multiple doses, long-term therapy in inexpensive, potent, forms with improved bioavailability is needed. The provision of an administration method, embodied by a dosage form that addresses FDC bioavailability concerns, that will allow patients to safely treat themselves and enhance their compliance with the anti-TB regimen is a significant health care development, particularly in developing countries where access to doctors, clean syringes, sterile needles, and sophisticated treatments are few and far between.

As mentioned, the major route of drug administration is through the oral cavity. This route provides the greatest comfort and convenience of dosing. In addition to avoiding the patient discomfort associated with the parentral route, the accidental overdosing of the drug can be corrected by withdrawing the unabsorbed drug from the stomach. An anti-TB dosage form that can be orally administered once daily would be optimal for patient compliance[42]. In addressing oral bioavailability concerns, chemical modification or pro drug formation may well be successfully implemented to alter the pharmacokinetics of RIF and INH. Prodrug strategies have successfully improved the oral bioavailability of numerous compounds. In many cases, this involves masking a polar group by esterification to increase lipophilicity and enhance the extent of absorption from the gastrointestinal tract. After absorption, the ester is enzymatically hydrolysed to release the parent drug.

RIF was developed in the Dow-Lepetit Research Laboratories (Milan, Italy) as part of an extensive program of chemical modification of the rifamycins, the natural metabolites of *Nocardia mediterranei* as the hydrazone with N-amino-N'-methyl piperazine that was the most active in the oral treatment of infections in animals and, after successful clinical trials, was introduced into therapeutic use in 1968[74]. To date, no form of RIF has been widely clinically applied that significantly improves on its oral bioavailability. There is little solubility advantage associated with polymorphic forms, which are in consequential from a clinical and regulatory point of view[75]. A piperine composition for the improvement of gastrointestinal absorption and systemic utilization of nutrients and nutritional supplements comprising an extract from the fruit of *Piper* containing a minimum of 98% of pure alkaloid piperine, has been added to multi-drug formulations for the treatment of TB and leprosy. A formulation, containing RIF, INH and PYZ and the said composition, has been tested in human volunteers (Indian Patent No.1232/DEL/89). In the majority of cases, the comparative levels and peak concentration of the drugs in the presence of piperine were higher.

The applicability of these results to bioavailability enhancement, which aims to lower dosage levels and shorten the treatment course, is apparent but presently cost prohibitive in developing countries. INH was synthesized in 1912 from ethyl isonicotinate and hydrazine by Meyer and Malley as part of their doctoral work in Prague. In 1945, its anti-TB properties were elucidated when nicotinamide was discovered to have anti-TB effects. Being a pro-drug itself, activated through endogenous mycobacterial catalysis, various additional chemical modifications have been investigated to alter INH pharmacokinetics.

Gianolla et al (1992)[76] attached various acyl groups to the amine (-NH2) function of INH for improved lipophilicity and afforded good yields in prodrugs, which were characterised by spectroscopic and analytical methods. Crooks et al (2004)[77] proposed the fabrication of an INH prodrug through the formation of covalent conjugates of INH with mono-di- and polyoxy alkanoic ortho alkanoic acids. The conjugation is purported to provide covalent compounds having a chemotherapeutic effect, with enhanced permeation of biological membranes, which remain intact until enzymatically cleaved.

As reported, Zhou et al (2005)[15] developed an ionizable prodrug of INH, INHMS, for sustained delivery of INH to alveolar macrophages. The charged prodrug was ion paired with two different hydrophobic cations: tetra pentyl ammonium-and tetra heptyl ammonium-bromide. The prodrug required loading into microparticles for realization of the targeted sustained effect. Prodrug formation is clinically relevant in altering *in vivo* disposition kinetics and in attaining sustained release, but developments have not necessarily addressed the deleterious RIF-INH interaction upon oral administration. Shishoo et al (2001)[3] have promoted the need for the development of a stable formulation containing the RIF-INH combination for differentiated GI release and have suggested enteric-coated tablets or alternative multilayered dosage forms.

In developing an oral modified-release system, cognizance must be taken of the increase in popularity of multiparticulate solid dosage forms (e.g. micro particles, nanoparticles, beads, pellets, granules) in the area of oral controlled drug delivery[78]. Formulation of an anti-TB dosage form as an oral multiparticulate drug-delivery system would furnish many biopharmaceutical advantages when compared with solid single-unit dosage forms in terms of a more even and predictable distribution and transportation in the gastrointestinal tract that is fairly independent of the nutritional state, predictable gastrointestinal transit time, less localized gastrointestinal disturbances and greater product safety; as well as having an application in the improvement of patient compliance. In view of the many benefits offered by multiple unit dosage forms, it is speculated that such systems are particularly useful for site-specific targeting within the gastrointestinal tract[79,80].

In order to manufacture an oral system as cheaply and efficiently as possible in developing countries, intrinsic drug delivery principles may be implemented, employing readily available polymeric and other formulatory excipients to segregate the delivery of the pure drugs. Agrawal et al (2004)[75] have indicated that RIF release in the acidic medium is critical for RIF bioavailability. There is thus the requisite to deliver RIF in a highly available form that will be readily absorbed from the gastric environment. The intended delivery system exemplifies the requirements of small intestinal INH delivery and immediate gastric availability of RIF.

### 3.7 Nanotechnology

Nanotechnology, the term derived from the Greek word Nano, meaning dwarf, applies the principles of engineering, electronics, physical and material science, and manufacturing at a molecular or submicron level. The materials at nano scale could be advice or a system or these could be supra molecular structures, complexes or composites[81].

An early promoter of nanotechnology, Albert Franks, defined it as ‘that area of science and technology where dimensions and tolerances are in the range of 0.1nm to 100nm. Nano technology is expected to make significant advances in the mainstream biomedical applications, including in the areas of gene therapy, drug delivery, imaging, and novel drug discovery techniques[82]. Nanotechnology is hailed as a new generation of technology with the potential to revolutionize many facts of the world we live in. This includes virtually all aspects of daily life, including health and health care, the manufacturing and use of materials and equipment, the environment and protection thereof. It is said to be able to massively increase manufacturing production at significantly reduced costs. Products of nanotechnology will be smaller, cheaper, lighter yet more functional and require less energy and fewer raw materials to manufacture. However, the 'revolution' will not happen overnight and very large investments in research and development will be required in the process.

Nanotechnology includes a bewildering array of activities including: molecular manufacturing, supra molecular and self assembly/organization; biomimicry; nanoparticles (e.g. Bucky balls and carbon nano tubes), nanospheres, nanocups and nanorods, nanobots (nanorobots), colloids, micelles, vesicles and nano-emulsions, clathrate complexes and intercalation compounds.

The National Science Foundation in the USA predicts that the global marketplace for goods and services using nanotechnologies will grow to $1 trillion by 2015, and there are already over 500 products being sold that claim they are made with nanoscale or engineered nanomaterials. These include products like self-cleaning windows, automobile paint, sunscreens, and tennis rackets. In the future, a marriage of nano- and biotechnology will likely create a whole new generation of drugs, biomedical devices, and other solutions to some of our most challenging medical problems[83].

### 3.7.1 Nanotechnology in drug delivery

The development of delivery systems for small molecules, proteins and DNA has been impacted to an enormous degree over the past decade by nanotechnology, and has led to the development of entirely new and somewhat unpredicted fields. For the pharmaceutical industry, novel drug delivery technologies represent a strategic tool for expanding drug markets. The technology can address issues associated with current pharmaceuticals such as extending product life (line extension), or can add to their performance and acceptability, either by increasing efficacy or improving safety and patient compliance[84]. This technology is permitting the delivery of drugs that are highly water- insoluble or unstable in the biological environment.

Advantages of nano sizing of drugs has the potential to: Increase surface area, enhance solubility, increase rate of dissolution, increase oral bioavailability, more rapid onset of therapeutic action, decrease the dose needed, decrease fed/fasted variability and decrease patient to patient variability.

### 3.7.2 Significance of drug delivery and targeting

Although opportunities to develop nanotechnology based efficient drug delivery systems extend into all therapeutic classes of pharmaceuticals, the development of effective treatment modalities for the respiratory, central nervous system and cardiovascular disorders remains a financially and therapeutically significant need.

Many therapeutic agents have not been successful because of their limited ability to reach to the target tissue. In addition, the faster growth opportunities are expected in developing delivery systems for anti cancer agents, hormones and vaccines because of safety and efficacy shortcomings in their conventional administration modalities. For example, in cancer chemotherapy, cytostatic drugs damage both malignant and normal cells alike. Thus, a drug delivery strategy that selectively targets the malignant tumor is very much needed. Additional problems include drug instability in the biological milieu and premature drug loss through rapid clearness and metabolism.

Similarly, high protein binding of certain drugs such as protease inhibitors limits their diffusion to the brain and other organs. However, nanotechnology for drug delivery applications may not be suitable for all drugs, especially those drugs that are less potent because the higher dose of the drug would make the drug delivery system much larger, which would be difficult to administer.

### 3.7.3 Role of nanotechnology in tuberculosis

#### 3.7.3.1 Nanotechnology in diagnosis of tuberculosis

The diagnosis tools are required to meet the needs of the WHO's expansion of the Directly Observed Treatment Short-course, MDR and co-infection with HIV. In India, the country with the highest estimated number of TB cases, research is underway into the role nanotechnology can play in addressing such concerns. The Central Scientific Instruments Organization of India designed a nanotechnology-based TB diagnostic kit, which is currently in the clinical trials phase. This kit does not require skilled technicians for use and offers portability, efficiency, user-friendliness and availability for less than US$1. The research is also ongoing for an optical biosensor for rapid TB detection in the Medical Sciences division of the U.S. Department of Energy. Another group at RMIT University, in Australia, is conducting research into the application of novel tethered nanoparticles as low-cost, colour based assays for TB diagnosis[85].

### 3.7.3.2 Nanotechnology in treatment of tuberculosis

Treatments with improved sustained release profiles and bioavailability can increase compliance through reduced drug requirements and there in minimize MDR-TB. Chemotherapy of TB is complex due to the requirement of multi drug regimens that need to be administered over long periods. The poor patient compliance is the single most common reason for chemotherapy failure in TB[86].

The micro-encapsulation of pharmaceutical substances in biodegradable polymers used in controlled drug delivery has seen as an emerging technology. Carrier or delivery systems such as liposomes and microspheres have been developed for the sustained delivery of anti-TB drugs and have found better chemotherapeutic efficacy when investigated in animal models (e.g. mice)[5].

The following are among the important technological advantages of nanoparticles as drug carriers: high stability (i.e., long shelf life); high carrier capacity (i.e., many drug molecules can be incorporated in the particle matrix); feasibility of incorporation of both hydrophilic and hydrophobic substances; and feasibility of variable routes of administration, including oral administration and inhalation. These carriers can also be designed to enable controlled (sustained) drug release from the matrix[87].

### 3.7.4 Nanoparticles

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000nm. The drug is dissolved, entrapped, encapsulated or attached to a nanoparticle matrix. Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. In recent years, biodegradable polymeric nanoparticles, particularly those coated with hydrophilic polymer such as poly (ethylene glycol) (PEG) known as long-circulating particles, have been used as potential drug delivery devices because of their ability to circulate for a prolonged period time, target a particular organ, as carriers of DNA in gene therapy, and their ability to deliver proteins, peptides and genes[88].

The major goals in designing nanoparticles as a delivery system are to control particle size, surface properties and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen. Though liposomes have been used as potential carriers with unique advantages including protecting drugs from degradation, targeting to site of action and reduction of toxicity or side effects, their applications are limited due to inherent problems such as low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components and poor storage stability. On the other hand, polymeric nanoparticles offer some specific advantages over liposomes. For instance, they help to increase the stability of drugs/proteins and possess useful controlled release properties[89].

**The Advantages of using nanoparticles as a drug delivery system are**

- Particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting after parenteral administration.
- They control and sustain release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug so as to achieve increase in drug therapeutic efficacy and reduction in side effects.
- Controlled release and particle degradation characteristics can be readily modulated by the choice of matrix constituents. Drug loading is relatively high and drugs can be incorporated into the systems without any chemical reaction; this is an important factor for preserving the drug activity.
- Site-specific targeting can be achieved by attaching targeting ligands to surface of particles or use of magnetic guidance.
- The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc.

In spite of these advantages, nanoparticles do have limitations. For example, their small size and large surface area can lead to particle-particle aggregation, making physical handling of nanoparticles difficult in liquid and dry forms. In addition, small particles size and large surface area readily result in limited drug loading and burst release. These practical problems have to be overcome before nanoparticles can be used clinically or made commercially available. Table 4 illustrate the therapeutic applications of nanoparticles and their marketed products[89].

**Table 4 Therapeutic applications of nanoparticles**

| Applications | Material | Purpose |
|---|---|---|
| Cancer therapy | Poly (alkylcyanoacrylates) nanoparticles with anticancer agents, oligonucleotides. | Targeting, reduced toxicity, enhanced uptake of anti tumors agents, improved *in-vitro* and *in- vivo* stability. |
| Intercellular targeting | Poly (alkylcyanoacrylates) Polyester nanoparticles with anti- parasitic or antiviral agents. | Targeting reticuloendothelial System for Intra cellular infections. |
| Prolonged systemic circulation | Polyesters with adsorbed polyethylene glycols or pluronics or derivatized polyesters | Prolong systemic drug effect, avoid uptake by the reticuloendothelial system. |
| Vaccine adjuvant | Poly (methylmethacrylate) nanoparticles with vaccines (oral and intramuscular immunization) | Enhanced immune response, alternate acceptable adjuvant |
| Per oral absorption | Poly (alkyalcyanoacrylate) nanoparticle with proteins and therapeutic agents | Enhanced bioavailability, Protection from gastrointestinal enzymes |
| Ocular delivery | Poly (alkylmethacrylate) nanoparticles with steroids, anti-inflammatory agents, Anti bacterial agents for glaucoma | Improved retention of drug |
| DNA delivery | DNA-alginate nanoparticles, DNA –chitosan nanoparticles, PDNA-poly(DL-lactide-co glycolide) Nanoparticle | Enhanced delivery and significantly higher expression levels |
| Other Applications | Nanoparticles with adsorbed enzymes Nanoparticles with radioactive or contrast agents | Enzyme immunoassays radio imaging |

### 3.7.5 Pharmaceutical approaches to nanoparticulate system

Nanoparticles (NPs) belong to the class of multiphase systems in which one or more micro phases are dispersed in a continuous matrix of different composition or physical state. The main characteristic of colloidal dispersions is their extremely large interface area between the dispersed phase and the continuous phase. Colloidal dispersions are metastable or unstable, since minimization of interface free energy between two different phases is dictated by thermodynamic constraints. However, in some cases colloids display significant kinetic stability that prevents their aggregation. Hence, production of microparticles (MPs) and NPs relies essentially upon the chemical production of colloidal dispersions, their kinetic stabilization, and effective recovery of the final formulates. Polymeric materials are constituted by large molecules whose peculiar solution characteristics often allow for the preparation of stable and size-controlled colloidal dispersions, which in turn can be converted into NPs. In addition, several polymers can be used as stabilizers of colloidal dispersions, since they provide a surface coating of the meta stable micro phase, thus lowering its tendency to phase-aggregation. The common feature of all methods for the preparation of MPs and NPs is the externally-induced separation of at least two phases. This process is better known as coacervation, and it may be promoted by a number of different techniques.

#### 3.7.5.1 Solvent evaporation method

In this method, the polymer is dissolved in an organic solvent such as dichloromethane, chloroform or ethyl acetate, which is also used as the solvent f dissolving the hydrophobic drug. The mixture of polymer and drug solution is then emulsified in an aqueous solution containing a surfactant or emulsifying agent to form oil in water (o/w) emulsion. After the formation of stable emulsion, the organic solvent is evaporated either by reducing the pressure or by continuous stirring. Particle size was found to be reducing the pressure or by continuous stirring. Particle size was found to be influenced by the type and concentrations of stabilizer, homogenizer speed and polymerconcentration. In order to produce small particle size, often a high-speed homogenizationor ultrasonication may be employed[90].

#### 3.7.5.2 Spontaneous emulsification or solvent diffusion method

This is a modified version of solvent evaporation method. In this method, the water miscible solvent along with a small amount of the water immiscible organic solvent is used as an oil phase. Due to the spontaneous diffusion of solvents an interfacial turbulence is created between the two phases leading to the formation of small particles. As the concentration of water miscible solvent increases, a decrease in the size of particle can be achieved. Both solvent evaporation and solvent diffusion methods can be used for hydrophobic or hydrophilic drugs. In the case of hydrophilic drug, a multiple w/o/w emulsion needs to be aqueous phase[91].

#### 3.7.5.3 Polymerization method

In this method, monomers are polymerized to form nanoparticles in an aqueous solution. Drug is incorporated either by being dissolved in the polymerization medium or by adsorption onto the nanoparticles after polymerization completed. The nanoparticle suspension is then purified to remove various stabilizers and surfactants employed for polymerization by ultracentrifugation and re-suspending the particles in an isotonic surfactant-free medium. This technique has been reported for making poly butyl cyano acrylate or poly (alkyl cyano acrylate) nanoparticles. Nanocapsules formation and their particle size depend on the concentration of the surfactants and stabilizers used[92].

### 3.7.5.4 Production of nanoparticles using supercritical fluid technology

Conventional methods such a solvent extraction-evaporation, solvent diffusion and organic phase separation methods require the use of organic solvents which are hazardous to the environment as well as to physiological systems. Therefore, the supercritical fluid technology has been investigated as an alternative to prepare biodegradable micro and nanoparticles because supercritical fluids are environmentally safe. A supercritical fluid can be generally defined as a solvent at a temperature above its critical temperature, at which the fluid remains a single phase regardless of pressure. Supercritical CO2 (SC CO2) is the most widely used supercritical fluid because of its mild critical conditions (Tc = 31.10C, Pc = 73.8 bars), non-toxicity, non-inflammability, and low price. The most common techniques involving supercritical fluids are supercritical anti-solvent (SAS) and rapid expansion of critical solution. The process of SAS employs a liquid solvent, e.g. methanol, which is completely miscible with the supercritical fluid to dissolve the solute to be micronized; at the process conditions, because the solute is insoluble in the supercritical fluid, the extract of the liquid solvent by supercritical fluid leads to the instantaneous precipitation of the solute, resulting the formation of nanoparticles[93].

### 3.7.5.5 Polyelectrolyte complex (PEC)

Polyelectrolyte complex or self assemble polyelectrolyte is a term to describe complexes formed by self-assembly of the cationic charged polymer and plasmid

DNA. Mechanism of PEC formation involves charge neutralization between cationic polymer and DNA leading to a fall in hydrophilicity as the polyelectrolyte component self assembly. Several cationic polymers (i.e. gelatin, polyethylenimine) also possess this property. Generally, this technique offers simple and mild preparation method without harsh conditions involved. The nanoparticles spontaneously formed after addition of DNA solution into Chitosan dissolved in acetic acid solution, under mechanical stirring at or under room temperature. The complexes size can be varied from 50 nm to 700 nm[94].

### 3.7.5.6 Microemulsion method

Chitosan NP prepared by microemulsion technique was first developed by Maitra *et al* 1999) [95]. This technique is based on formation of chitosan NP in the aqueous core of reverse micellar droplets and subsequently cross-linked through glutaraldehyde. In this method, a surfactant was dissolved in N-hexane. Then, chitosan in acetic solution and glutaraldehyde were added to surfactant/hexane mixture under continuous stirring at room temperature. Nanoparticles were formed in the presence of surfactant. The system was stirred overnight to complete the cross-linking process, which the free amine groups of chitosan conjugate with glutaraldehyde. The organic solvent is then removed by evaporation under low pressure. The yields obtained were the cross-linked chitosan NP and excess surfactant. The excess surfactant was then removed by precipitation with $CaCl_2$ and then the precipitant was removed by centrifugation. The final nanoparticles suspension was dialyzed before lyophilization. This technique offers a narrow size distribution of less than 100 nm and the particle size can be controlled by varying the amount of glutaraldehyde that alters the degree of cross-linking. Nevertheless, some disadvantages exist such as the use of organic solvent, time-consuming preparation process, and complexity in the washing step.

### 3.7.5.7 Ionic gelation method

In the ionic gelation method, Chitosan (CS) is dissolved in aqueous acidic solution to obtain the cation of CS. This solution is then added drop wise under constant stirring to poly anionic tripolyphosphate (TPP) solution. Due to the complexation between oppositely charged species, CS undergoes ionic gelation and precipitates to form spherical particles. The method is schematically represented in Fig.3 However, TPP/CS micro particles formed have poor mechanical strength thus, limiting their usage in drug delivery

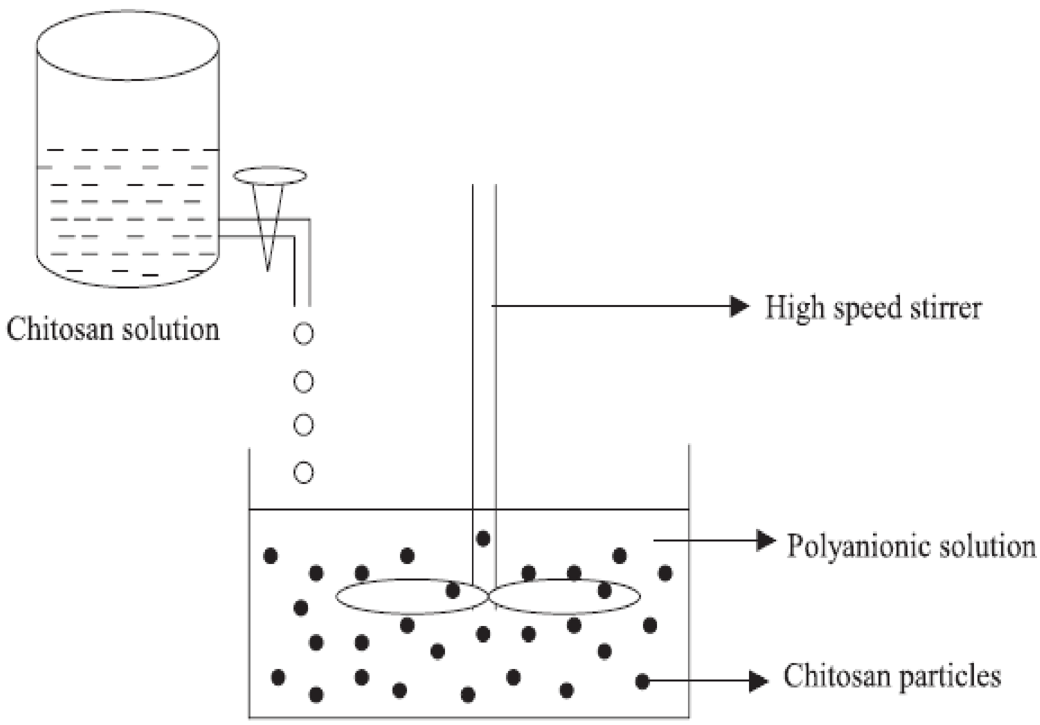



**Figure 3. Schematic representation of preparation of chitosan particulate systems by ionic gelation method.**

Insulin-loaded CS nanoparticles have been prepared by mixing insulin with TPP solution and then adding this to CS solution under constant stirring[96] .Two types of CS in the form of hydrochloride salt (SeacureR 210 Cl and ProtasanR 110 Cl), varying in their molecular weight and degree of deacetylation, were utilized for nanoparticle preparation. For both types of CS, TPP concentration was adjusted to get a CS/TPP ratio of 6:1. Chitosan nanoparticles thus obtained were in the size range of 300–400 nm with a surface charge ranging from ±54 to ±25 mV. Using this method, insulin loading was modulated reaching the values up to 55%. Efficiency of the method was dependent upon the deacetylation of CS, since it involves the gelation of protonated amino groups of CS.

There are many ongoing investigations, which demonstrate the improved oral bioavailability of peptide and protein formulations. Bioadhesive polysaccharide CS nanoparticles would seem to further enhance their intestinal absorption. Pan et al (2002)[97] prepared the insulin-loaded CS nanoparticles by ionotropic gelation of CS with TPP anions. Particle size distribution and zeta potential were determined by photon correlation spectroscopy. The ability of CS nanoparticles to enhance the intestinal absorption of insulin and the relative pharmacological bioavailability of insulin was investigated by monitoring the plasma glucose level of alloxan-induced diabetic rats after the oral administration of various doses of insulin-loaded CS nanoparticles. The positively charged, stable CS nanoparticles showed particle size in the range of 250–400 nm. Insulin association was up to 80%. The in vitro release experiments indicated initial burst effect, which is pH-sensitive. The CS nanoparticles enhanced the intestinal absorption of insulin to a greater extent than the aqueous solution of CS in vivo. After administration of 21 I.U/kg insulin in the CS nanoparticles, hypoglycemia was prolonged over 15h. The average pharmacological bioavailability relative to s.c. injection of insulin solution was up to 14.9%.

Xu and Du[98] have studied different formulations of CS nanoparticles produced by the ionic gelation of TPP and CS. TEM indicated their diameter ranging between 20 and 200 nm with spherical shape. FTIR confirmed tripolyphosphoric groups of TPP linked with ammonium groups of CS in the nanoparticles. Factors that affect the delivery of bovine serum albumin (BSA) as a model protein have been studied. These include molecular weight and deacetylation degree of CS, concentrations of CS and BSA, as well as the presence of polyethylene glycol (PEG) in the encapsulation medium. Increasing molecular weight of CS from 10 to 210 kDa, BSA encapsulation efficiency was enhanced nearly twice. The total release of BSA in phosphate buffered saline pH 7.4 in 8 days was reduced from 73.9% to 17.6%. Increasing deacetylation degree from 75.5% to 92% promoted the encapsulation efficiency with a decrease in release rate. Encapsulation efficiency decreased greatly by increasing the initial concentration of BSA and CS. Higher loading capacity of BSA enhanced the BSA release from nanoparticles. However, adding PEG hindered the BSA encapsulation and increased the release rate.

Ko et al (2002)[99] prepared CS micro particles with TPP by the ionic cross-linking method. Particle sizes of TPP-CS micro particles varied from 500 to 710 Am with drug encapsulation efficiencies more than 90%.Morphologies of TPP-CS micro particles have been examined by SEM.As the pH of TPP solution decreased and molecular weight of CS increased, micro particles acquired better spherical shape having smooth surface. Release of felodipine as a model drug was affected by the preparation method. Chitosan micro particles prepared at lower pH or higher concentration of TPP solution resulted in a slower release of felodipine. With a decreasing molecular weight and concentration of CS solution, the drug release increased. The release of drug from TPP-CS microparticles decreased when the cross-linking time was increased.

### 3.7.6 Oral administration of nanoparticle-based TB drugs

Stability of nanoparticles offers the possibility of oral administration. The fate of nanoparticles in the gastrointestinal tract has been investigated in a number of studies[100,101,102]. In general, the uptake of nanoparticles occurs as follows: (*1*) by transcytosisvia M cells, (*2*) by intracellular uptake and transport via the epithelial cells lining the intestinal mucosa, (*3*) by uptake via Peyer's patches. Pandey and colleagues[48] demonstrated that the nanoparticles provided sustained release of the anti-TB drugs and considerably enhanced their efficacy after oral administration.

Three frontline drugs, rifampin (RMP), isoniazid (INH), and pyrazinamide (PZA) were co encapsulated in poly (lactide-co-glycolide) (PLG) nanoparticles. After a single oral administration of this formulation to mice, the drugs could be detected in the circulation for 4 d (RMP) and 9 d (INH and PZA); therapeutic concentrations in the tissues were maintained for 9 to 11 d. In contrast, free (unbound) drugs were cleared from the plasma within 12 to 24hrs after administration.

Treatment of *M. tuberculosis*–infected mice with the nanoparticle-bound drugs (five oral doses every10th day) resulted in complete bacterial clearance from the organs. Free drugs were able to produce bacterial clearance only after daily administration of 46 doses. Similar efficacy of the nanoparticle-bound drugs was also observed in guinea pigs[103]. At the same time, incorporation in microparticles was less effective: their drug-loading capacity was lower as well as the plasma half-life of the bound drugs[15,53].

The behavior of polymeric nanoparticles in the gastrointestinal tract is influenced by their bioadhesive properties; adhesion of nanoparticles to the mucosa enhances the absorption of the associated drug, thus increasing its bioavailability. Thus, lectin shave been shown to improve mucoadhesion of the drug due to the bio recognition of the lectin-grafted carriers by glycosylated structures in the intestine[105]. Accordingly, the efficacy of PLG-based formulations of anti-TB drugs was further improved by covalent attachment of wheat germ agglutinin[105].

Oral administration of wheat germ agglutinin–coated PLG nanoparticles loaded with RIF, INH, and PZA in mice produced considerably extended serum half life :detectable RIF serum levels were observed for 6 to 7 d and INH and PZA for 13 to 14 d (vs. 4–6 d and 8–9 d for non modified nanoparticles). All three drugs were present in lungs, liver, and spleen for 15 d. The lectin-modified formulations produced bacterial clearance in these organs after three oral doses administered every 14 d (45 daily doses of free drugs). As suggested by the authors, the prolonged circulation of drugs encapsulated in wheat germ agglutinin–grafted nanoparticles might be attributed to the fact that lectins enhance prolonged adhesion of the particles to the intestinal surface to allow (1) an increase in the time interval available for absorption and (2) a localized increase in the concentration gradient between luminal and aerosal sides of the membrane. Table 5 illustrate release of drug and therapeutic efficacy of the nanoparticle-based formulations of the first-line antituberculous drugs[87].

**Table 5 Drug release and therapeutic efficacy of the nanoparticle-based formulations of the first-line antituberculous drugs — Rifampicin, Isoniazid, and Pyrazinamide**

| Delivery system | Animal model | Administration route | Duration of drug release (d) | | Regimen producing sterilizing effect in lungs and spleen |
|---|---|---|---|---|---|
| | | | Plasma | Organs | |
| PLG nanoparticles | Mice | Oral | 6-9 | 9-11 | 5 doses ever 10 d |
| | Mice | Subcutaneous | 32 | 36 | Single injection |
| | Guinea Pigs | Aerosol | 4-9 | Up to 10 d (each drug) | 5 doses every 10 d |
| | Guinea Pigs | Oral | 4-9 | Up to 10 d (each drug) | 5 doses every 10 d |
| | Guinea Pigs | Oral | 7-13 | Up to 15 d (each drug) | 3 doses fortnightly |
| Lectin – functionalized PLG nanoparticles | Guinea Pigs | Aerosol | 6-14 | Up to 15 d (each drug) | 3 doses fortnightly |
| Solid lipid nanoparticles | Guinea Pigs | Aerosol | 5 | 7 | 7 doses weekly |

PLG –poly (lactide-co-glycolide), The drug-to-polymer ratio is 1:1 for each drug

### 3.7.7 Criteria for ideal polymeric carriers for nanoparticles and nanoparticle delivery systems[106]

**Polymeric carriers**

- Easy to synthesize and characterize
- Inexpensive
- Biocompatible
- Biodegradable
- Non-immunogenic
- Non-toxic
- Water soluble

**Nanoparticle delivery systems**

- Simple and inexpensive to manufacture and scale-up
- No heat, high shear forces or organic solvents involved in their preparation process.
- Reproducible and stable
- Applicable to a broad category of drugs; small molecules, proteins and poly nucleotides
- Ability to lyophilize
- Stable after administration
- Non-toxic

Table 6, 7 illustrate polymers used for the preparation of nanoparticles and polymeric nanoparticles for antimicrobial drug delivery [107,108].

**Table 6 Polymers used for the preparation of nanoparticles**

| **Technique** | **Candidate Drug** | **Polymer used** |
|---|---|---|
| Heat denaturation and cross linking in w/o emulsion | Hydrophilic | Hydrophilic Albumin Gelatin |
| Desolvation and cross linking in water | Hydrophilic and protein affinity | Hydrophilic Albumin, Gelatin |
| Cross – linking in water | Hydrophilic and protein affinity | Hydrophilic Alginates and chitosan |
| Polymer precipitation in an organic solvent | Hydrophilic | Hydrophilic Dextran |
| Emulsion polymerization | Hydrophilic | Hydrophilic Poly(alkylcyanoacrylates) |
| Interfacial w/o polymerization | Hydrophilic | Hydrophilic Poly(alkylcyanoacrylates) |
| Solvent extraction evaporation | Hydrophilic and Hydrophilic Soluble in polar solvent | Polyester Poly (Lactic acid), poly(Caprolactone) |
| Solvent displacement | Hydrophilic and Hydrophilic Soluble in polar solvent | Polyester Poly (Lactic acid), poly(lactide-co-glycolide) |
| Salting out | Soluble in polar solvent | Polyester Poly (Lactic acid), poly(lactide-co-glycolide) |

**Table 7 Polymeric nanoparticles for antimicrobial drug delivery**

| Formulation | Drug | Targeted microorganism | Activity |
|---|---|---|---|
| Poly(D, Lactide)PLA Nanospheres | arjunglucoside | Leishmania | Reduced toxicity |
| Polylactic co glycolic acid(PLGA)nanoparticles | Phosphorothioate antisense oligoneucleotide | HIV | Protection of olegonucleotide from degradation |
| Poly(ethylene oxide) (PEO)-modified poly (epsilon-carprolactone) (PCL) nanoparticle | Saquinavir | HIV | 1)Protect the drug from cytochrome O metabolish<br>2)bypass P-gp efflux pump |
| Alginate nanoparticles | Rifampicin, isoniazid, pyrazinamide and ethambutol. | Mycobacterium tuberculosis | 1)High drug payload<br>2) improved pharmacokinetics<br>3) High therapeutics efficacy |
| Poly-lactide-co-glycolide(PLG) nanoparticles | Rifampicin, isoniazid, pyrazinamide and ethambutol. | Mycobacterium tuberculosis | 1)Enhanced bioavailability Improved pharmacodynamics |
| Polaxamer 188 coated poly(epsilon-caprolactone)(PCL) nanosphere | Amphotericin B | Candida albicans | Lower in vivo toxicity due to reduced accumulation in kidney and liver |
| Polyethylene glycol (PEG)-PLA nanocapsules | Halofantrine | Plasmo diamberghe | Prolonged circulation half life |
| Poly(isohexylcyanoacrylate) (PIHCA) nanospheres | Primaquine and ampicillin | Leishmania donovant, Salmonella typhynurium and listeria monocytogenes | Particle itself exhibits antimicrobial activity |
| Glycosylated polyacrylate nanoparticles | Beta-lactum ciprofloxacin | Staphylococcus aereus and Bacillus anthracis | Improved bioavailability, Higher therapeutic efficacy |

### 3.8 Ultrasonication

Ultrasonication is a common tool for the preparation and processing of polymer nanoparticles. It is particularly effective in breaking up aggregates and in reducing the size and polydispersity of nanoparticles[109]. The physical stability and in vivo distribution of nanoparticles are affected by their mean size, polydispersity, and surface charge density[110]. Despite the wide-spread applications of ultrasonication in nanotechnology, its effects on chitosan nanoparticles are not well understood, although there have been several reports on the ultrasonication of the chitosan polymer.

It is generally agreed that ultrasonication causes main chain scissions at the 1,4-glycosidic bond[111] without affecting the degree of deacetylation (DD) of chitosan samples. The process has, therefore, been conveniently applied to produce chitosan samples of lower molecular weights of the same DD[112,113,114]. It was hypothesized that ultrasonic-mediated depolymerization of chitosan would influence the properties of the chitosan nanoparticles. The complexity of the chitosan nanoparticle system may further render it more vulnerable to chemical modifications by ultrasonication. High-intensity ultrasonication produces acoustic cavitation, which generates hot spots of short lifetimes with intense local heating of ~5000$^{\circ}$C, pressures of ~1000 atm, and heating and cooling rates above 1010 K/s[115]. These, together with free-radical formation, may mediate redox reactions and intra molecular regroupings in the samples [116]. Cavitation also generates rapid streaming of solvent molecules around the cavitation bubble, as well as shock waves during bubble collapse, which in turn generate very large shear forces[115]. In addition, rare fractions and compressions of the liquid media can cause dispersive (particle separation) and coagulative (collision and adhesion of particles) phenomena, respectively[117].

Ultrasonic treatments were administered using an ultrasonic probe with diameter of 3mm and a 130 Whigh-intensity ultrasonic processor (VC130, Sonics and Materials Inc., USA) operating at 20 kHz. The converter was made of piezoelectric lead zirconate crystals. Samples (24 ml) in glass universal bottles (Beatson and Co., UK) were equilibrated to 25°C and ultrasonicated under continuous mode at ambient conditions. The probe was immersed 4 cm into the sample during ultrasonication, which was carried out at specified amplitudes (20, 40, 60, 80) over durations of 2–10 min. For simplicity, the ultrasonication conditions are denoted as *AxTy* where *x* represents the amplitude and *y* the duration. Amplitudes of 20, 40, 60, and 80 corresponded to intensities of approximately 14, 42, 70, and 99 W/cm2, respectively, the intensity calculated by taking the difference between the output Watts delivered into the sample and in air, divided by the area of the probe tip. Ultrasonication produced similar heating effects in the chitosan solution and chitosan nanoparticle samples. In both cases, temperature increased linearly from 25 to 45°C for samples ultrasonicated for 5 min at increasing amplitudes from 0 to 80, and from 25 to 41°C for samples ultrasonicated at the amplitude of 40 with increasing duration from 0 to 10 min. Treated samples were cooled to ambient temperature and analyzed immediately. Some samples were lyophilized (FD3, Dynavac Engineering, Australia) before analysis.

### 3.9 Freeze drying of nanoparticles

Protective excipients, such as carbohydrates, are widely used in freeze-drying to ensure redispersibility and to avoid aggregation or size changes of nanoparticles[118]. Glucose and lactose were evaluated as cryo- and lyo protectants for the L- PLA nanoparticles because these nanoparticles could not survive during the drying process without protectants. Even the smallest tested amount of glucose (weight ratio glucose: nanoparticles 1:4) was found to protect the nanoparticles, although the appearance of the dried material was translucent and sticky, and its redispersibility was poor. When lactose was used as a protectant, it enhanced the appearance of the cake (the dried material) as a white powder, eligible for a freeze-dried formulation. Redispersion of the nanoparticle was possible, but as a form of visible aggregates. Further freeze-thawing experiments revealed that already the freezing step (with lactose) destroyed the particles. Next, the two carbohydrates were used together to combine the cryoprotective functionality of glucose and the lyoprotective functionality of lactose. The best result, prolonged Tyndall effect (opalescence in the dispersion) after redispersion of the dried formulation and good quality nanoparticles were obtained, when the amount of lactose was double the amount of glucose. The weight ratios of glucose and lactose to the nanoparticles were 1:2 and 1:1, respectively. Additionally, when an extra stabilizer, Tween 80, was used during the nanoparticle preparation or during the redispersion, the freeze-dried cake could be redispersed more easily with increased stability (prolonged Tyndall effect).

The good cryoprotective results with glucose probably arise from its ability to bind water molecules to the amorphous phase which it forms during the freezing step. Part of the water in the frozen glucose remained non-frozen (even 32% w/w). That water acted as a plasticizer and as a spacing matrix reducing the pressure of ice crystals against the nanoparticles and preventing harmful aggregation caused by freeze concentration, respectively. At the same time, insufficient cryoprotective function of lactose derived most likely from its lower water binding activity. However, as a combination with glucose, lactose reduced the amount of water to a level where the interaction of glucose with water was reduced and, thus, the formation of ice crystal was slightly promoted. This enabled sufficient evaporation of water during the drying and formation of a proper cake. Tween 80 improved the freeze-drying result as it acted as a steric stabilizer and increased the hydrophilicity of the nanoparticles. A hydrophilic surface enhances the redispersion properties of the freeze-dried nanoparticles[119,120].

This technique involves the freezing of the nanoparticle suspension and subsequent sublimation of its water content under reduced pressure to get a free flowing powdered material following advantages are cited for the freeze drying of nanopartilces:

- Prevention from degradation and/or solubilization of the polymer.
- Prevention from drug leakage, drug desorption and/or drug degradation.
- Easy to handle and store and helps in long-term prevention/conservation of nanoparticles.
- Readily dispersible in water without modification in their physicochemical properties.

### 3.10 Chitosan

Chitosan [(1 → 4) 2-amino 2-deoxy -d-glucan] is a linear polyamine with a high ratio of glucosamine to acetyl-glucosamine units. The percentage of glucosamine units in the polymer is known as its degree of deacetylation (DD)[121]. Protonation of the amino group allows the polymer to be solubilized in aqueous acids and to interact with negatively charged materials[122]. It is this functional group that enables the formation of chitosan nanoparticles by crosslinking and desolvation with cationic salts[105]. Chitosan nanoparticles are attractive non-viral carriers for the delivery of peptides, proteins, oligonucleotides, and plasmids[123]. They have the capacity to protect sensitive bioactive macromolecules from enzymatic and chemical degradation in vivo and during storage[125] and to facilitate the transport of charged macromolecules across absorptive epithelial cells[125].

Chitosan possesses some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic, and inexpensive. Furthermore, it possesses positively charge and exhibits absorption enhancing effect. These properties render Chitosan a very attractive material as a drug delivery carrier. In the last two decades, Chitosan nanoparticles have been extensively developed and explored for pharmaceutical applications[106].

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases (conventional dosage forms), only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biochemical properties. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of a drug while reducing its toxic side effects *in vivo* is a challenging task.

One approach is the use of colloidal drug carriers that can provide site specific or targeted drug delivery combined with optimal drug release profiles. The idea of using submicron drug delivery systems for drug targeting was conceived and developed after Paul Ehrlich originally proposed the idea of tiny drug-loaded magic bullets over a hundred year ago. Among these carriers, liposomes and micro/nanoparticles have been the most extensively investigated. Liposomes present some technological limitations including poor reproducibility and stability, and low drug entrapment efficiency. Nevertheless, several low molecular weight drugs are now commercially available which employ this technology. Polymeric nanoparticles, which possess a better reproducibility and stability profiles than liposomes, have been proposed as alternative drug carriers that overcome many of these problems. The details of Chitosan-based drug delivery systems prepared by different methods for various kinds of drugs were given in Table 8[126].

**Table 8 Chitosan-based drug delivery systems prepared by different methods for various kinds of drugs**

| Type of system | Method of preparation | Drug |
|---|---|---|
| Tablets | Matrix coating | Diclofenac sodium, Pentoxyphylline, Salicylic acid, Theophylline, Propranolol HCL. |
| Capsules | Capsule shells | Insulin, 5-amino salicylic acid. |
| Microsphere/ Microparticles | Emulsion cross- linking | Theophylline, cisplatin, pentazocine, phenobarbitone, insulin, 5-fluorouracil, declofenac sodium, griseofulvin, aspirin, diphtheria toxoid, pamidronate, suberolbis phosphonate, mitoxantrone, progesterone. |
| | Coacervation/ precipitation<br>Spray drying | Prednisolone, interleukin-2, propranol-HCL<br>Cimetidine, famotidine, nizatidine, vitamin D-2, diclofenacsodium, ketoprofen, metoclopromide-HCL, bovine serum albumin, ampicillin, cetylpyridinium chloride, oxytetracycline, betamethazone. |
| | Ionic gelation<br>Seiving method | Felodipine,Clozapine.<br>Gadopentetic acid. |

| | | |
|---|---|---|
| Nanoparticles | Emulsion-droplet coalescence<br>Coacervation/ precipitation<br>Ionic gelation | DNA, doxorubicin<br>Indulin, riein, bovin serum albumin, cyclosporine A,<br>Doxorubicin. |
| | Reverse micellar method | Adriamycin, nifedipine, bovin serum albumin,s albutamol sulfate, lidocaine-HCL, Riboflavin. |
| Beads | Coacervation/ precipitation | Isosorbide dinitrate, cholorhexidine gluconate, trypsin, Granulocyte-macrophages, colony stimulating factor, acyclovir. |
| Films | Solution casting | riboflavine, testosterone, progesterone, beta-oestradiol, Chlorpheniraminemaleate, aspirin, theophylline, caffeine. |
| Gel | Cross-linking | lidocaine-HCL, hydrocortisone acetate,5-flourouracil. |

### 3.10.1 Characteristics of nanoparticles on drug delivery

Table 9 illustrate different parameters and characterizaton methods of nanopaticles[89]

**Table 9 Different parameters and characterizaton methods of nanopaticles**

| Parameter | Characterization method |
|---|---|
| Particle size and distribution | Photo correlation spectroscopy (PCS)<br>Laser defractometry<br>Transmission electron microscopy(TEM)<br>Scanning electron microscopy(SEM)<br>Mercury porositometry |
| Charge determination | Laser Doppler anemometry<br>Zeta potentiometer |
| Surface hydrophobicity | Water Doppler Anemometry<br>Rose Bengal (dye) binding<br>Hydrophobic interaction chromatography<br>X-ray photoelectron spectroscopy |
| Chemical analysis of surface | Static secondary ion mass spectroscopy |
| Carrier-drug interaction | Differential scanning calorimetry (DSC) |
| Nanoparticles dispersion stability | Critical flocculation temperature (CFT) |
| Release profile | *In vitro* release characteristics under physiologic and sink conditions |
| Drug stability | Bioassay of drug extracted from nanoparticles / chemical analysis of drug |

## 3.11 Characterization of nanoparticles

### 3.11.1 Morphology

Nanoparticles possess a variety of shapes and their names are characterized by their different shapes. For example, there are nanospheres that are spherical, nanoreefs, nanoboxes, nanoclusters, nanotubes etc. These shapes or morphologies sometimes arise spontaneously as an effect of a templating or directing agent during synthesis for example during miscellar emulsions or anodized alumina pores, or from the iniate crystallographic growth patterns of the materials themselves.

The morphologies of nanoparticles help serve their various purposes such as long carbon nanotubes being used to bridge an electrical junction. Amorphous particles usually adopt a spherical shape or nanospheres and anisotropic microcrystalline whiskers correspond to their particular crystal shape. Small nanoparticles usually form clusters. These may be of various shapes like rods, fibers, and cups etc. The study of fine particles is called micromeritics.

Controlling the morphology of nanoparticles is of key importance for exploiting their properties for their use in several emerging technologies. Optical filters and bio-sensors are among the many applications that use optical properties of gold nanoparticles and it requires anisotropy of the particle shape as larger shapes produce greater plasmon losses.

Despite the great importance of the morphology of nanoparticles, it is generally not well characterized and practically never controlled. However, this is of prime importance. For example, this is important in magnetic devices where well-defined magnetization axes and switching fields are required to store or to process information[127].

### 3.11.2 Particle size

Particle size and size distribution are the most important characteristics of nanoparticle systems. They determine the *in vivo* distribution, biological fate and targeting ability of nanoparticle systems. In addition they can also influence the drug loading, drug release and stability of nanoparticles.

Many studies have demonstrated that nanoparticles of sub-micron size have a number of advantages over microparticles as a drug delivery system[128]. Generally nanoparticles have relatively higher intracellular uptake compared to microparticles and available to wider range of biological targets due to their small size and relative mobility. 100nm nanoparticles had a 2.5 fold greater uptake than 1µm microparticles and 6 fold greater uptakes than 10µm microparticles in a CACO-2 cell line[129]. In a subsequent study, the nanoparticles penetrated throughout the sub mucosal layers in a rat in situ intestinal loop model, while microparticles were predominantly localized in the epithelial lining. It was also reported that nanoparticles can cross the blood-brain barrier following the opening of tight junctions by hyper osmotic mannitol, which may provide sustained delivery of therapeutic agents for difficult-to-treat diseases like brain tumors. Tween 80 coated nanoparticles have been showed to cross the blood-brain barrier. In some cell lines, only submicron nanoparticles can be taken up effectively but not the larger size microparticles. The recent literature shows ophthalmic nanosuspension that proves to be boon for drugs that exhibit poor soluble in lachrymal fluid.

Drug release is affected by particle size. Smaller particles have larger surface area; therefore, most of the drug associated would be at or near the particle surface, leading to fast drug release. The particle surface, leading to fast drug release.

Whereas, larger particles have large cores which allow more drug to be encapsulated and slowly diffuse out[130]. Currently, the fastest and most routine method of determining particle size is by photon-correlation spectroscopy or dynamic light scattering. Photon-correlation spectroscopy requires the viscosity of the medium to be known and determines the diameter of the particle by Brownian motion and light scattering properties[131]. The results obtained by photon-correlation spectroscopy are usually verified by scanning or transmission electron microscopy (SEM or TEM).

### 3.11.3 Surface properties of nanoparticles

When nanoparticles were administered intravenously, they are easily recognized by the body immune systems, and are then cleared by phagocytes from the circulation. Apart from the size of nanoparticles, their surface hydrophobicity determines the amount of adsorbed blood components, mainly proteins. This is turn influences the *in vivo* fate of nanoparticles. Binding of these opsonins onto the surface of nanoparticles called opsonization acts as a bridge between nanoparticles and phagocytes. The association of a drug to conventional carriers leads to modification of the drug biodistribution profile, as it is mainly delivered to the mononuclear phagocytes system such as liver, spleen, lungs and bone marrow. Indeed, once in the blood stream, surface non-modified nanoparticles are rapidly opsonized and massively cleared by the macrophages of mononuclear phagocytes system rich organs. Generally, it is IgG, compliment $C_3$ components that are used for recognition of foreign substances, especially foreign macromolecules.

Hence, to increase the likelihood of the success in drug targeting by nanoparticles, it is necessary to minimize the opsonized and to prolong the circulation of nanoparticles *in vivo*. This can be achieved by surface coating of nanoparticles with biodegradable copolymers with hydrophilic segments such as polyethylene glycol (PEG) polyethylene oxide, polyoxamer, poloxamine and polysorbate 80 (Tween 80).

The zeta potential of a nanoparticle is commonly used to characterize the surface charge property of nanoparticles[132]. It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the *nanocapsule* or adsorbed onto the surface.

### 3.11.4 Drug-polymer interactions

Drug loading can be performed during the preparation of nanoparticles or by adsorbing/absorbing in preformed particles. Within the particle-forming polymer, drug can be present as a solid solution (individual drug molecules) or as a solid dispersion (amorphous/crystalline drug). It can be adsorbed on the particle surface or bound chemically within the nanoparticles .The preparation process can also modify the crystal structure of the drug. The polymer is usually amorphous or semi-crystalline. Differential scanning calorimetry (DSC), (powder) X-ray diffractometry (XRPD) and FTIR are commonly used techniques to reveal the physicochemical state and possible interactions of the drug and the polymer in pharmaceutical micro- and nanoparticles. Polymer MW is determined e.g. by size exclusion chromatography (SEC) the term gel permeation chromatography (GPC) is interchangeably used.

DSC detects phase transitions such as glass transition, (exothermic) crystallization and (endothermic) melting: the nanoparticle sample is heated and changes in heat flow, compared to reference, are registered. Crystallinity/amorphicity properties are obtained from XRPD analysis when diffraction pattern of the x-ray from the sample is determined as a function of scattering angle. In FTIR, a vibrational spectrum, characteristics for a given crystal structure, is obtained.

Absence of the drug melting peak and diffraction peaks of the crystal structure of the drug in DSC thermo gram and XRPD pattern, respectively, are usually signs of amorphous or molecularly dispersed drug within the polymer .It can also indicate that the amount of drug is lower than the detection limit of the instrument. Drug polymer interactions (e.g. plasticizing effect of drug on polymer) or polymorph change of the drug can be detected as peak shifts in DSC thermogram, band shifts in FTIR spectra or as new reflections in XRPD pattern. Correspondingly, smoothened XRPD pattern, increased cold crystallization exotherms (DSC) or some band shifts to higher wave numbers (FTIR) indicate increased amorphicity of the polymer[133].

### 3.11.5 Drug loading

Ideally, a successful nanoparticulate system should have a high drug-loading capacity thereby reduce the quantity of matrix materials for administration. Drug loading can be done by two methods

- Incorporating at the time of nanoparticles production (incorporation method)

- Absorbing the drug after formation of nanoparticles by incubating the carrier with a concentrated drug solution (adsorption /absorption technique) [137].

Drug loading and entrapment efficiency very much depend on the solid-state drug solubility in matrix material or polymer (solid dissolution or dispersion), which is related to the polymer composition, the molecular weight, the drug polymer interaction and the presence of end functional groups (ester or carboxyl). The PEG moiety has no or little effect on drug loading. The macromolecule or protein shows greatest loading efficiency when it is loaded at or near its isoelectric point when it has minimum solubility and maximum adsorption  For small molecules, studies show the use of ionic interaction between the drug and matrix materials can be a very effective way to increase the drug loading[135].

### 3.11.6 *In-vitro* drug release

To develop a successful nanoparticulate system, both drug release and polymer biodegradation are important consideration factors. In general, drug release rate depends on: (1) solubility of drug; (2) desorption of the surface bound/adsorbed drug; (3) drug diffusion through the nanoparticle matrix; (4) nanoparticle matrix erosion/degradation; and (5) combination of erosion/diffusion process. Thus solubility, diffusion and biodegradation of the matrix materials govern the release process. In the case of nanospheres, where the drug is uniformly distributed, the release occurs by diffusion or erosion of the matrix under sink conditions. If the diffusion of the drug is faster than matrix erosion, the mechanism of release is largely controlled by a diffusion process. The rapid initial release or 'burst' is mainly attributed to weakly bound or adsorbed drug to the large surface of nanoparticles[136]. It is evident that the method of incorporation has an effect on release profile. If the drug is loaded by incorporation method, the system has a relatively small burst effect and better sustained release characteristics. If the nanoparticle is coated by polymer, the release is then controlled by diffusion of the drug from the core across the polymeric membrane. The membrane coating acts as a barrier to release, therefore, the solubility and diffusivity of drug in polymer membrane becomes determining factor in drug release. Furthermore release rate can also be affected by ionic interaction between the drug and addition of auxiliary ingredients. When the drug is involved in interaction with auxillary ingredients to form a less water soluble complex, then the drug release can be very slow with almost no burst release effect; whereas if the addition of auxillary ingredients e.g., addition of ethylene oxide-propylene oxide block copolymer (PEO-PPO) to chitosan, reduces the interaction of the model drug bovine serum albumin (BSA) with the matrix material (chitosan) due to competitive electrostatic interaction of PEO-PPO with chitosan, then an increase in drug release could be observed[137].

Various methods which can be used to study the *in vitro* release of the drug are: (1) side-by-side diffusion cells with artificial or biological membranes; (2) dialysis bag diffusion technique; (3) reverse dialysis bag technique; (4) agitation followed by ultracentrifugation/centrifugation; (5) Ultra-filtration or centrifugal ultra-filtration techniques. Usually the release study is carried out by controlled agitation followed by centrifugation. Due to the time-consuming nature and technical difficulties encountered in the separation of nanoparticles from release media, the dialysis technique is generally preferred.

From the above mentioned literature, it is clearly understood that nanoparticulate approach is beneficial to overcome the disadvantage of poor bioavailability of drugs.Ascorbic acid has been reported to stabilize rifampicin from degradation in the dissolution medium or in the plasma sample; however, its usefulness in protecting rifampicin from degradation in the acidic environment of stomach has not been addressed. Ascorbic acid administration (1000mg/day p.o) is recommended in the clinical practice to suppress tuberculous infection by activating the immunity. Among the polymers, chitosan has been reported advantageous for development of nanoparticles used in the treatment of chronic infections like TB. Based on the above considerations, the present study aimed to develop chitosan loaded rifampicin-ascorbic acid nanoparticles and evaluate their physicochemical, in-vitro dissolution stability, in-vitro diffusion and in-vivo bioavailability in comparision with their respective control formulations.

## 3.12 Background of the study

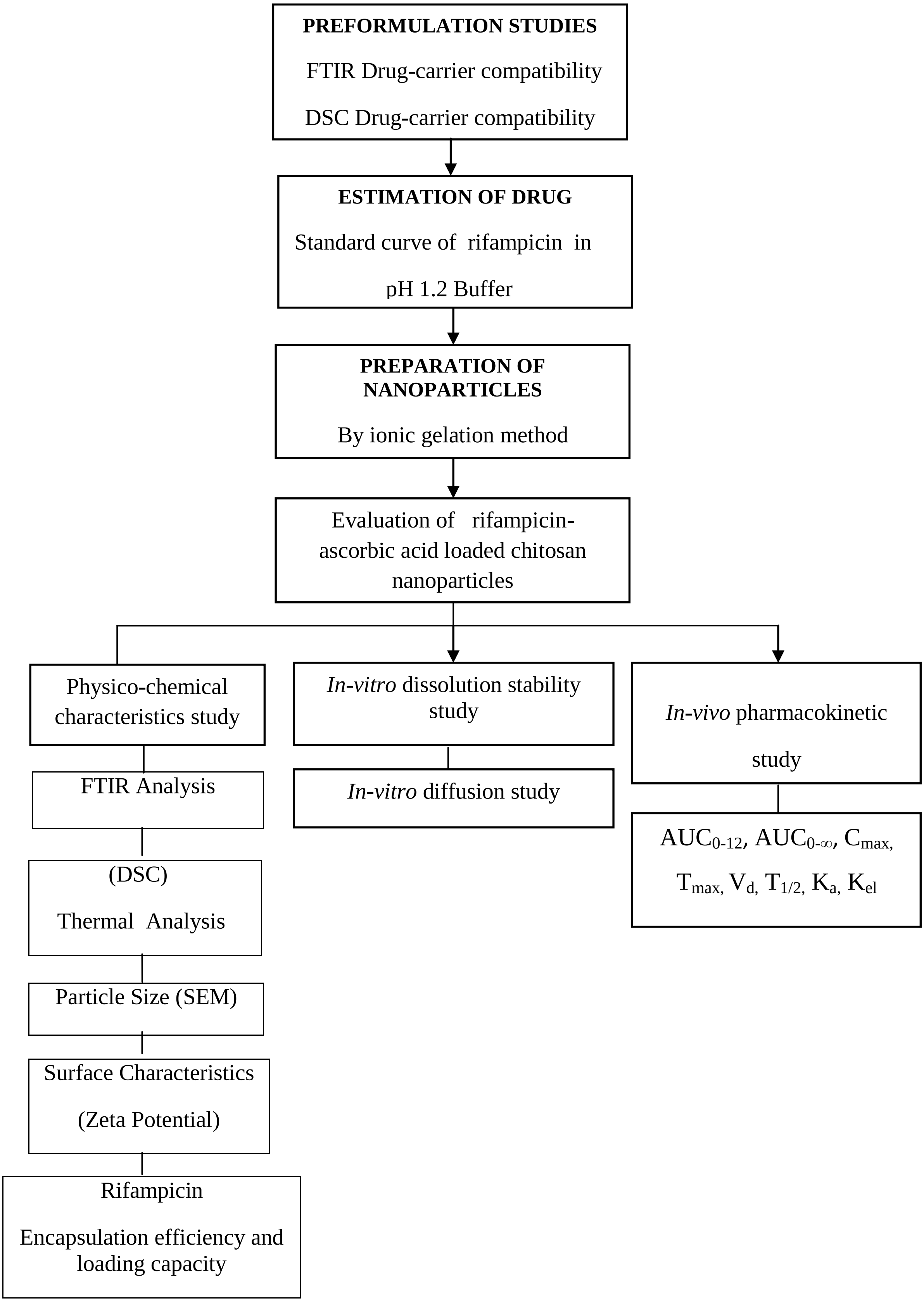
Bioavailability of rifampicin from fixed dose products containing isoniazid, pyrazinamide, and ethambutol remains challenging owing to its degradation in the acidic environment of the stomach following oral administration. Previous studies reveal that different approaches such as controlled release system, microspheres and nanoparticles were attempted to improve the efficacy of anti TB drugs in animal model; however no approach has yielded beneficial results. It has been well documented that any attempt that protects rifampicin degradation with improved absorption in the stomach is most beneficial to effectively control TB.

Recently nanoparticle delivery of drugs that are poorly bioavailable has assumed significance because nanosizing of drugs has the potential to improve surface area, enhance solubility, increase rate of dissolution, increase oral bioavailability, more rapid onset of therapeutic action, decrease the dose needed, decreased fed/fasted variability and decrease patient-patient variability. It has also been documented that ascorbic acid used to stabilize rifampincin in this dissolution media as well as in the plasma sample against degradation. Furthermore daily intake of ascorbic acid/day is also recommended to improve immunity against tuberculous infection. The possible effect of ascorbic acid in stabilizing rifampicin against gastric degradation has not been established.

Polymer plays a critical role in the development of ascorbic acid nanoparticles with desired characteristics. It has been recommended that natural polymer such as chitosan is advantageous for development of nanoparticles for chronic infection such as TB. Besides chitosan has also been reported to improve absorption of drugs because of its mucoadhesive property which provides prolonged contact of the drug with the biological membrane at the site of delivery.

Based on the above factors the present study was directed to investigate whether ascorbic acid and nanoparticulate delivery of rifampicin together can address the poor bioavailability of rifampicin in the presene or absence of isoniazid.

# 4. PLAN OF WORK



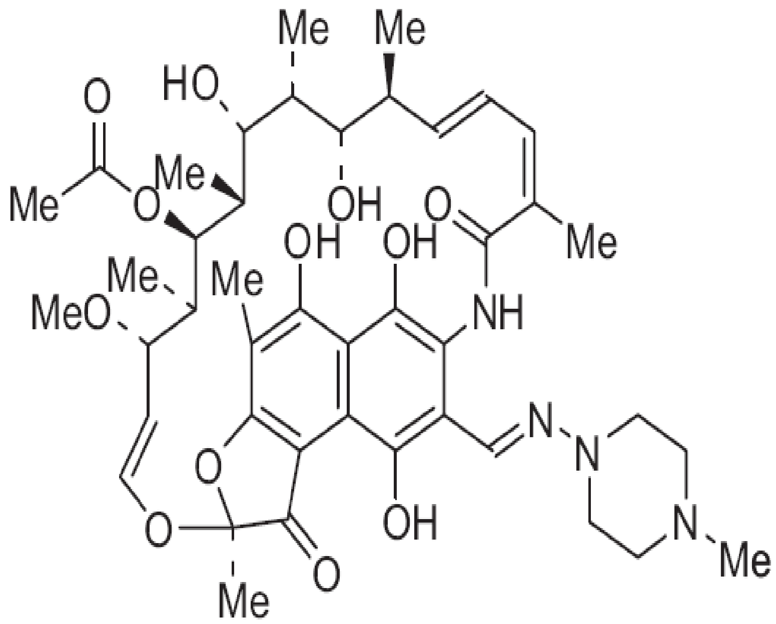
# 5. DRUG PROFILE

## 5.1 RIFAMPICIN

**Rifampin** is a bactericidal antibiotic drug of the rifamycin group[138]. It is a semi synthetic compound derived from *Amycolatopsis rifamycinica*[139] (formerly known as *Amycolatopsis mediterranei* and *Streptomyces mediterranei*).

**Molecular formula:** $C_{43}H_{58}N_4O_{12}$

**Chemical structure**



**Generic name:**

5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl- 8-[N-(4-methyl-1-piperazinyl)formimidoyl]-2,7epoxypentadeca[1,11,13]trienimino)naphtho[2,1-b]furan-1, 11(2H)-dione 21-acetate.

**Molecular weight:** 822.94

**Solubility:**

Freely soluble in chloroform and DMSO; soluble in ethyl acetate, methanol, tetrahydrofuran; slightly soluble in acetone, water, carbon tetrachloride

**Polarity:** (Log P) 3.719

**Acidity/ basicity :** pKa 1.7 for the 4-hydroxy and pKa 7.9 for the 3-piperazine nitrogen

**Stability :** Very stable in DMSO; rather stable in water

**Melting point:** 183ºC

**Optimal human dosage**

Dose 10 mg/kg, in a single daily administration, not to exceed 600 mg/day, oral or i.v

***In vitro* potency For *M. tuberculosis***: H37Rv, MIC is 0.4 mg/ml[138].

**Mechanism of action**

Rifampicin inhibits the essential *rpoB* gene product b-subunit of DNA dependent RNA polymerase activity, acting early in transcription[139]. It is thought to bind to the ß-subunit, close to the RNA/DNA channel, and physically blocks the transit of the growing RNA chain after nucleotides have been added[139,140].

**Spectrum of activity**

Rifampicin is bactericidal with a very broad spectrum of activity against most grampositive and some gram-negative organisms (including *Pseudomonas aeruginosa*) and *M. Tuberculosis*. Rifampicin has clinical efficacy against a wide variety of organisms, including *Staphylococcus aureus*, *Legionella pneumophila*, Group-A *Streptococcus*, *Brucella* spp., *Haemophilus nfluenzae*, and *Neisseria meningitidis*, as well as *in vitro* activity against penicillin-resistant *S. pneumoniae*, *N. gonorrhoeae*, *Chlamydia trachomatis*, *H. ducreyi*, and many gram-negative rods. Due to rapid emergence of resistant bacteria it is restricted to treatment of mycobacterial infections, where the customary use of combination drugs delays resistance development, and the treatment of asymptomatic meningococcal carriers[141].

**Pharmacokinetics of Rifampicin**

**Absorption**

Rifampicin is well absorbed from the gastrointestinal tract, with peak plasma levels achieved within 1 to 4 h after oral administration, although food may delay its absorption[142,143]. After intravenous dose administration the plasma levels in adults are about 9 μg/ml (300mg infusion) over 30 min. after infusion.

**Distribution**

Rifampicin readily diffuses into most organs, tissues, bones and body fluids, including exudates into tuberculosis infected lung cavities[144]. Therapeutic concentrations are achieved in saliva reaching 20% of serum concentrations. High concentrations appear in the lachrymal glands and tears. The urine is coloured orange to brick red. It is reported that rifampicin is highly protein bound to an extent of 84-91%[145]. Tissue distribution occurs at a relatively fast rate. At physiological pH only about 25% of the drug is ionized while the molecule as a whole is lipid soluble. Levels of rifampicin in the cerebrospinal fluid are approximately one tenth of those achieved in the blood, although this may be increased in inflammatory states [146,147].

**Metabolism**

The principal pathways of metabolism of rifampicin involve desacetylation and hydrolysis. Desacetylation at the C-25 position results in a more polar and equally active compound, 25- desacetyl rifampicin (DAR), with increased capacity for biliary excretion. Depending on the dose of rifampicin, one-third to one-eighth may be excreted in the bile, either as a 25-DAR or as unchanged rifampicin. The unchanged rifampicin is reabsorbed, creating an enterohepatic circulation, whereas the 25-DAR is poorly absorbed[148]. The half-life of rifampicin is 3-5 h. Rifampicin, stimulates its own metabolism in liver and the biliary excretion of desacetyl rifampicin [149]. On first dose administration on an empty stomach of 300 mg rifampicin, the serum concentration curves are similar to those following intravenous dosing, indicating little presystemic metabolism, but repeated administration induces hepatic endoplasmic reticular enzymes. Rifampicin induces certain cytochrome P450s, mainly 3A4 isozyme. The bioavailability of the active, orally administered rifampicin decreased from 93% after the first single oral dose to 68% after 3 weeks of oral and intravenous rifampicin therapy. This is attributed to both, an increased hepatic metabolism and an induction of a prehepatic “first-pass” effect resulted from multiple rifampicin doses [150].

**Excretion**

Rifampicin is mainly eliminated in bile, gets reabsorbed and undergoes enterohepatic circulation. Amount excreted in urine increases with increasing doses and upto 30% of dose of 900 mg may be excreted in urine, about half of it within 24 h[145,151,152]. About 40% is excreted in bile. About 60-65% dose appears in feces. Within 24 h, 3-30% of unchanged drug and active metabolite get excreted in urine (600 mg single dose oral administration). 6-15% of dose is excreted in urine and 15% of dose appears as active metabolite (25-DAR) in urine. 7% of dose is excreted as inactive 3-FRSV[152].

**Drug-Drug interactions**

Rifampicin induces certain cytochrome P450s, mainly 3A4 isozyme. The rifampicin dose of 600 mg/day was established partly to limit the CYP3A induction potential[153]. The drug affects the metabolism of the following drugs: acetaminophen, astemizole, carbamazepine, corticosteroids, cyclosporin, dapsone, ketoconazole, methadone, phenobarbital, phenytoin, quinidine, terfenadine, theophylline, verapamil and warfarin[149].

**Adverse effects of Rifampicin**

**Human adverse reactions**: Hepatitis and serious hypersensitivity reactions including thrombocytopenia, hemolytic anaemia, renal failure have been reported. Asymptomatic elevations of serum transaminase enzymes, increase in serum bile acids and bilirubin concentrations can occur. Marked elevation of serum alkaline, phosphatase and bilirubin suggests rifampicin liver toxicity.

Cardiovascular: Hypotension and shock.

Respiratory: Shortness of breath.

CNS: Rare cases of organic brain syndrome have been reported (i.e. confusion, lethargy, ataxia, dizziness and blurring of vision).

**Gastrointestinal:** Nausea, vomiting, diarrhoea. Rifampicin causes orange-red staining of all body fluids [154,141].

**Indications**

The primary indications for rifampicin are for treatment of tuberculosis (pulmonary andextrapulmonary lesions) and for leprosy. It has recently been used for brucellosis[141].

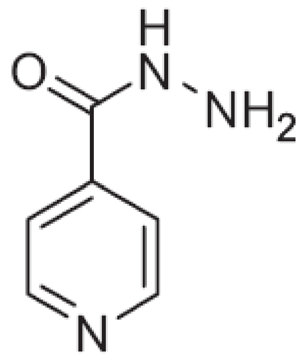
**Contraindications**

Rifampicin is contraindicated in known cases of hypersensitivity to the drug. It may be contraindicated in pregnancy (because of teratogenicity noted in animal studies and since the effects of drugs on foetus have not been established) except in the presence of a disease such as severe tuberculosis. It is contraindicated in alcoholics with severely impaired liver function and with jaundice [141].

## 5.2 ISONIAZID

**Molecular formula**: $C_6H_7N_3O$

**Chemical structure**



**Generic Name:** Isonicotinic acid hydrazide; isonicotinoyl hydrazine; isonicotinyl Hydrazine.

**Molecular weight:** 137.14

**Solubility:** Soluble in water (~14% at 25ºC, ~26% at 40ºC) ethanol: (~2%at 25ºC), boiling ethanol (~10%), chloroform (~0.1%). Practically insoluble in ether, benzene.

**Polarity:** (Log P) 0.64

**Acidity / basicity:** pH of a 1% aqueous solution 5.5 to 6.5

**Stability:** Very stable in DMSO; rather stable in water

**Melting point:** 171.4ºC

**Optimal human dosage**

5 mg/kg for adults, 10-20 mg/kg for children. Adult dosing generally 300 mg capsule administered orally, once daily; or 15 mg/kg up to 900 mg/day, two or three times/week, ideally dose administered 1 h before or 2 h after a meal. Concomitant administration of pyridoxine ($B_6$) recommended for malnourished patients,

adolescents, and those predisposed to neuropathy (e.g.diabetic).Can also be given intramuscularly or intravenously[29].

***In-vitro* potency For *M. tuberculosis:*** H37Rv, MIC is 0.025 mg/ml.

**Mechanism of action**

Isoniazid is a prodrug activated by catalase-peroxidase hemoprotein, KatG. Isoniazid inhibits InhA, a nicotinamide adenine dinucleotide (NADH)-specific enoyl-acyl carrier protein (ACP) reductase involved in fatty acid synthesis[141].

**Spectrum of activity**

Isoniazid is a bactericidal agent active against organisms of the genus *Mycobacterium*, specifically *M. tuberculosis*, *M. bovis* and *M. kansasii*. Isoniazid is bactericidal to rapidly-dividing mycobacteria, but is bacteristatic if the mycobacterium is slow growing. Isoniazid is highly specific, being active against only a subset of the mycobacteria and largely ineffective against other microorganisms; this is in part due to several unusual aspects of metabolism, exemplified in *M. tuberculosis*, including unusually high KatG activity and a defective drug efflux mechanism[155].

**Pharmacokinetics of isoniazid**

**Absorption**

Isoniazid is readily absorbed when administered either orally or parentrally. Peak plasma concentrations of 3-8 μg/ml develop 1-2 h after fasting dose of 300 mg orally. Aluminium containing antacids may interfere with the absorption of isoniazid[156,157].

**Distribution**

Isoniazid diffuses readily into all body fluids and cells. Isoniazid is not considered to be bound appreciably to plasma proteins. The concentration of the drug is initially higher in the plasma and muscle than in the infected tissue, but the latter retains the drug for long time in quantities well above those required for bacteriostatis [158,159].

**Metabolism**

The plasma half-life of isoniazid ranges from 1-4 hrs, those who are fast acetylators because of genetic variations, having short half-lifes. The primary metabolic route is acetylation of isoniazid to acetylisoniazid by N-acetyltransferase, form in the liver and small intestine. Acetyl isoniazid is then hydrolyzed to isonicotinic acid and monoacetylhydrazine, isonicotinic acid is conjugated with glycine to isonicotinyl glycine and monoacetylhydrazine is further acetylated to diacetylhydrazine. Some unmetabolized isoniazid is conjugated to hydrazones. The metabolites of isoniazid have no tuberculostatic activity and are non-toxic[157].

**Excretion**

Elimination of isoniazid from the body is dependent upon its genetically controlled rate of acetylation. Excretion is primarily renal. From 75% to 95% of dose of isoniazid is excreted in the urine within 24 h, mostly as metabolites. In patients with normal renal function, over 70% of a dose appears in the urine in 24 h. Of this amount, 93% of the isoniazid excreted in urine in fast acetyltors in the form of N-acetylisoniazid and 63% in slow acetylators as N-acetylisoniazid[159]. Small amounts of drug are also excreted in faeces[157].

**Drug-drug interactions**

Isoniazid interacts with the cytochrome P-450 system, especially CYP2E1, where it shows a biphasic inhibition induction; it causes increases in serum concentrations of various drugs, especially phenytoin and carbamazepine, increases the effects of warfarin and theophylline, inhibits metabolism of benzodiazepines, and inhibits monoamine oxidase and histaminases. Isoniazid should not be administered with food, as studies have shown that this significantly reduces its bioavailability[141].

**Adverse effects of isoniazid**

**Central Nervous System (CNS) effects:** Peripheral neuropathy is the most common CNS related toxic effect. It is dose-related, occurs most often in the malnourished and in those predisposed to neuritis (e.g., alcoholics and diabetics), and is usually preceded by paraesthesias of the feet and hands. The incidence is higher in “slow acetylators”. Other neurotoxic effects, which are uncommon with conventional doses, are convulsions, toxic encephalopathy, optic neuritis and atrophy, memory impairment and toxic psychosis.

**Hepatic effects:** Isoniazid does carry a specific warning of the potential for liver toxicity. Liver toxicity and hepatitis risks are increased with concomitant use of carbamazepine, phenobarbital, rifampicin, and alcohol abuse. Elevated serum transaminase (SGOT, SGPT), bilirubinaemia, bilirubinuria, jaundice, and occasionally severe and sometimes fatal hepatitiscan occur with normal dosing regimens. The common prodromal symptoms of hepatitis are anorexia nausea, vomiting, fatigue, malaise, and weakness. Mild hepatic dysfunction, evidenced by mild and transient elevation of serum transaminase levels appears in the first 1-3M of treatment but can occur at any time during therapy. In most instances enzyme levels return to normal, and generally there is no necessity to discontinue medication during the period of mild serum transaminase elevation. The frequency of progressive liver damage increases with age. It is rare in persons under 20, but occurs in up to 2.3% of those over 50 years of age[141,155].

**Gastrointestinal effects:** Nausea, vomiting, epigastric distress and dark urine can occur but are rare.

**Haematological effects:** agranulocytosis; hemolytic, sideroblastic, or aplastic anaemia, thrombocytopenia; and eosinophilia can occur[141].

**Endocrine and metabolic:** Pyridoxine deficiency, pellagra, hyperglycaemia, acidosis and gynecomastia can occur[155].

**Hypersensitivity:** Fever, skin rashes, lymphadenopathy and vasculitis can occur[160].

**Indications**

The primary indications for isoniazid is for the treatment of tuberculosis (pulmonary and extrapulmonary lesions[157].

**Contraindications**

Isoniazid is contraindicated in known cases of hypersensitivity to the drug. It is contraindicated in alcoholics with severely impaired liver function and with jaundice[155].

# 6. POLYMER PROFILE

## 6.1 CHITOSAN

Chitosan is a natural cationic biopolymer consequent commencing the hydrolysis of chitin. One perceptible improvement of this substance is that it can be obtained from ecologically sound natural sources, namely crab and shrimp shell wastes. Together with chitin, Chitosan is well thought-out the second most profuse polysaccharide subsequent to cellulose. However contrasting cellulose, the employ of Chitosan as an excipient in pharmaceutical formula is a pretty new development. But Chitosan has been widely premeditated in the biomedical field and has been found to be highly biocompatible. In addition to the good biocompatibility of Chitosan and the abundance of natural sources of the material, Chitosan has a number of enviable properties that put together study of it attention-grabbing[161].

**Origin of Chitosan**

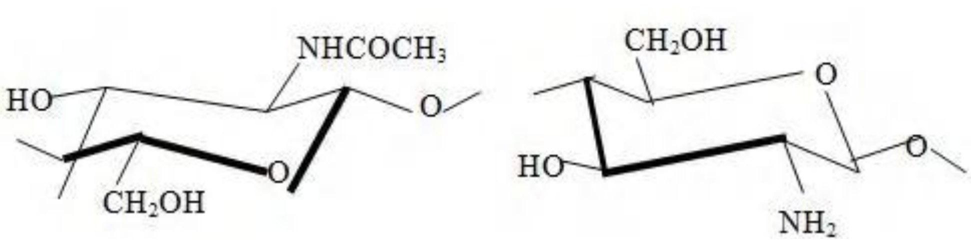
Chemist always plays molecules. Chitin did not escape from it. Chemist did not spare chitin, the polymer either and made Chitosan. Chitin is the primary structural component of the outer skeletons of crustaceans, and of many other species such as molluscs, insects and fungi. Chitosan is most commonly obtaining from crustacean chitin, from crab and shrimp shells wastes. Chitin accounts for approximately 70% of the organic components in such shells. It is a reinforcing material, which occurs in three polymorphic forms, a-, b- and g-chitin. Where hardness in needed a-chitin is found, where flexibility is required b- and g-chitin occur. Chitin is inert in aqueous environment. Chitosan is prepared from chitin to obtain a more reactive polymer. The term Chitosan is used when chitin could be dissolved in weak acid. When chitin is heated in a strong solution of sodium hydrochloride (>40%) at high temperature (90-120$^{o}$)[161].

**Scope for chitin/ chitosan production in India**

The international shrimp industry from harvest through various processingoperations produces a vast amount of potentially recoverable proteinaceous by-productsin the form of shrimp heads and shells which is one of the major raw materials for chitin/ Chitosan production. Shells of other crustaceans viz. crabs, lobsters, squilla, and cuttle fish bones also could be profitably utilized.In India, it is estimated that more than one lakh tonne of shrimp processing wasteis being wasted annually which could be gainfully utilized for manufacturing chitin ahigh value industrial product. Another raw material for chitin is squilla. It is estimated that a potential of around 50,000 tonne of squilla is available of which nearly 5,000 tonne is being thrown back into the sea. This is an important trawl by catch especially in Mangalore and could be used for chitin/chitosan production. Crab shells and lobster shells are also raw materials for chitin/chitosan production. The estimated availability of crab shells is 30,000 - 40,000 tone in the Indian waters[161].

**Chemistry**

Chitosan (Poly [-(1,4)-2-amino-2-deoxy-D-glucopiranose] ) is a natural carbohydrate polymer prepared by the partial N-deacetylation of chitin .Chitin is isolated from shells of crustacean( for example shrimp, crab and lobster ) by treating the shells with 2.5 N NaOH at 75 $^{o}C$ and with 1.7 N HCl at room temperature for 6 hours Deacetylation can be done by alkaline treatment or by enzymatic reaction. The alkaline deacetylation is carried out by treating chitin with NaOH at high temperature. The degree of deacetylation increases with increasing temperature or NaOH concentration.




**Structure of Chitosan**

**Important properties of chitosan**

- Medical grade micronised Chitosan is biodegradable, non allergic, haemostatic, non toxic and wound healing accelerator.
- Chitosan films are flexible, tough, transparent, clear and oxygen permeable with good tensile strength.
- Chitosan could be used to make single and biopolymer membranes, non woven fabrics and sponges for surgical applications.
- It is resistant to alkali, digestive enzymes and urine.
- Chitosan also could be cross linked.

**Solubility**

Chitosan is soluble in organic acids like formic, acetic, prop ionic. oxalic, malonic, succinic, adipic, lactic, pyruvic, malic, tartaric and citric acid[162].

**Process**

**Technology**

Central Institute of Fisheries Technology (CIFT) Kochi has the distinction of perfecting the technology for chitin and Chitosan production in the country. The institute is imparting training to entrepreneurs who are interested in setting up such units. They also provide technical support on a turnkey basis.

**Raw material**

Dried/wet shells of prawns, squilla, crabs, lobsters etc., could be utilized. The shells thus used should be thoroughly free from sand and extraneous matter, so as to reduce the ash content of the final product to less than 2%.

**Deproteinisation**

The shells are boiled with 3% sodium hydroxide for 30minutes in a mild steel vessel to remove protein stuck to head and shell. The boiled raw material is allowed to cool and it is washed with water to remove all traces of alkali (could be tested with a pH paper).

**Demineralisation**

The deproteinised shells are transferred to a mild steel vessel lined with fiber glass and is treated with 3% hydrochloric acid. This is kept for 30minutes with occasional stirring till the reaction is complete. The excess acid is decanted and the residue is washed till the pH is normal.

**Removal of water**

Excess water is removed using a screw press till the moisture is below 60%. The product thus obtained is called chitin.

**Deacetylation of Chitin**

It is the process of conversion of chitin to Chitosan. Chitin is heated at 90-95 0 C for about one and a half hour with 40% caustic soda in amild steel vessel. Excess alkali is drained off and the mixture is washed with water several times till it is free from alkali. Eighty percent of the alkali, thus removed could be reused in subsequent cycles.

**Drying**

The above product is sun dried for 6-8 hours or in drier till the moisture content is less than 5%. Care should be taken not to exceed the drier temperature beyond 60 $^{0}$ C. chitosan thus obtained is in the form of flakes.

**Powdering and Packing**

The Chitosan flakes obtained could be powdered and packed in lots of 10, 20, and 25 kg in HDP/ Polyethylene lined non woven sacks in a dry place. Chitin can be stored for one year whereas chitosan can be stored for nearly three months only.

**Yield**

Chitin represent 14-27% and 13-15% of the dry weight of shrimp and crab processing waste respectively and squilla yields 15% chitin. On a conservative basis the yield is estimated as dry raw material chitin 14% by wt Chitosan 10% by wt[163].

**Product characterization**

- Chitosan can be described in general by the following parameters:
- Degree of deacetylation in %,
- Dry matter in %,
- Ash in %,
- Protein in %,
- Viscosity in Centipoises,
- Intrinsic viscosity in ml/g,
- Molecular weight in g/mol, and
- Turbidity in NTU units

All of these parameters can be adjusted to the application for which chitosan is being used. The deacetylation is very important to get a soluble product. In general, the solubility of heteroglucans are also influenced by the distribution of the acetyl groups, the polarity and size of the monomers, distribution of the monomers along the chain, the flexibility of the chain, branching, charge density, and molecular weight (50,000 to 2,000,000 Da) of the polymer. Viscosity (10 to 5000 cp) can be adjusted to each application by controlling the process parameters[164].

**Chemical and biological properties of chitosan**

- Cationic polyamine Biocompatibility
- High charge density at pHs < 6.5 Natural polymer
- Adheres to negatively charged surfaces Safe and non-toxic
- Forms gels with polyanions Haemostatic
- High molecular weight linear polyelectrolyte
- Biodegradable to normal body constituents
- Viscosity, high to low Bacteriostatic / Fungistatic
- Chelates certain transitional metals Spermicidal
- Amiable to chemical modification Anticancerogen
- Reactive amino/hydroxyl groups Anticholestermic

**Specification of pharmaceutical-grade chitosan parameters description**

- Appearance (powder or flake) White or yellow
- Particle size < 30µm
- Viscosity A ≤ 5 cps
- Density between 1.35 to 1.4 g/cm3
- PH 6.5 to 7.5
- Moisture content > 10%
- Ash value > 2%
- Mater insoluble in water ≤ 0.5%

- Degree of deacetylation 66 to 99.8%
- Heavy metal (Pb) < 10 ppm
- Heavy metal (As) < 10 ppm
- Protein content < 0.3%
- Loss on drying ≤ 10 %
- Glass transition temperature 203 0C

**Uses of chitosan**

**Clarification and purification**

The property of long chain molecules of dissolved Chitosan to wrap the solid particles suspended in liquids and to bring them together and agglomerate makes it suitable as a coagulant aid. It is used in treatment of sewage effluents, purification of drinking water etc.

**Chromatography**

The presence of free amino acid hydroxyl groups in Chitosan is a good chromatographic support.

**Paper and textiles**

The high molecular weight, poly cationic linear film forming and hydrogen bonding ability makes chitosan an ideal polymer applicable in the paper industry. The chelating ability, adhesive property and ionic bond forming characteristic of chitosan find potential application in textiles. Fabrics seized with chitosan have good stiffness, improved dye uptake, added luster and improved laundering resistance.

**Photography**

Due to the resistance of chitosan to abrasion, optical characteristic film forming ability and behaviors with silver complexes chitosan has important application in photography.

**Food and nutrition**

Chitosan supplemented chick feed and fish feed improved the weight gain in chicken and fish[165].

**Agriculture**

It has potential application in agriculture such as germination and culturing to enhance self protection against pathogenic organisms in plants and suppress them in soil, to induce chitinase activity, in encapsulation of fertilizers, in liquid fertilizers and incontrolled release of herbicides.

**Medical and pharmaceutical uses**[166]

- Disintegrant
- Bioadhesive polymer Diluents in direct compression of tablets.
- Binder in wet granulation
- Slow-release of drugs from tablets and granules
- Drug carrier in microparticle systems
- Films controlling drug release
- Preparation of hydrogels, agent for increasing viscosity in solutions.
- Wetting agent, and improvement of dissolution of poorly soluble drug substances
- Site-specific drug delivery (e.g. to the stomach or colon)
- Absorption enhancer (e.g. for nasal or oral drug delivery)
- Biodegradable polymer (implants, microparticles)
- Carrier in relation to vaccine delivery or gene therapy.
- Bacteriostatic agent
- Enzyme immobilization
- Film / membrane for dialysis

- Artificial skull sponge for mucosal haemostatic agent wound dressing
- Anti cholesteremic material
- Anti sore composition
- Antibillirubinemia agent
- Preparation of self regulated drug delivery system
- Sustained release / direct compression
- Buccal and sublingual drug delivery
- Ophthalmic drug delivery
- Gastrointestinal drug delivery
- Transdermal drug delivery

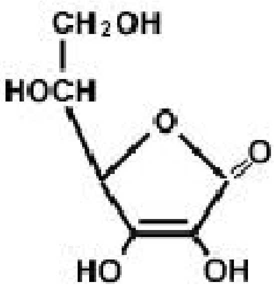
# 7. EXCIPIENT PROFILE

## 7.1 ASCORBIC ACID

**Category:** Water Soluble Vitamin

**Empiricalformula:**$C_6H_8O_6$

**Structure:**



**Chemical name:** L-ascorbic acid

**Molecular weight:** 176.1

**Melting point: A**bout 190°C (with decomposition)

**Colour:** White to slightly yellowish crystalline powder

**Taste:** Slight acidic taste.

**Solubility:** Freely soluble in water; sparingly soluble in alcohol; insoluble in chloroform, in ether, and in benzene.

**Dosage and Administration**

Ascorbic acid (vitamin c) is usually administered orally. When oral administration is not feasible or when malabsorption is suspected, the drug may be administered IM, IV, or subcutaneously. When given parentrally, utilization of the vitamin reportedly is best after IM administration and that is the preferred parentral route.

The average protective dose of vitamin C for adults is 70 to 150 mg daily. In the presence of scurvy, doses of 300 mg to 1 g daily are recommended. However, as much as 6 g has been administered parentrally to normal adults without evidence of toxicity.

**Toxicity**

- Unpleasant diarrhea[167]
- Gastrointestinal disturbances[168]
- Hyperoxaluria [169]
- Haemolysis [170]

**Mechanism of antioxidants**

Ascorbic acid is a mild reducing agent. For this reason, it degrades upon exposure to oxygen, especially in the presence of metal ions and light. It can be oxidized by one electron to a radical state or doubly oxidized to the stable form called dehydroascorbic acid.

Ascorbate usually acts as an antioxidant. Typically it reacts with oxidants such reactive oxygen species, such as the hydroxyl radical formed from hydrogen peroxide. Such radicals are damaging to animals and plants at the molecular level due to their possible interaction with nucleic acids, proteins, and lipids. Sometimes these radicals initiate chain reactions. Ascorbate can terminate these chain radical reactions by electron transfer. Ascorbic acid is special because it can transfer a single electron, owing to the stability of its own radical ion called "semidehydroascorbate". dehydroascorbate. The net reaction is:

$$RO\bullet + C_6H_7O_6^- \rightarrow ROH + C_6H_6O_6\text{-}\bullet.$$

The oxidized forms of ascorbate are relatively unreactive, and do not cause cellular damage.

However, being a good electron donor, excess ascorbate in the presence of free metal ions can not only promote, but also initiate free radical reactions, thus making it a potentially dangerous pro-oxidative compound in certain metabolic contexts.

**Functions**

- Antioxidant which helps defend cells from the effects of smoke, pollution and other highly reactive substances called free radicals
- Controls blood cholesterol levels
- Converts folic acid into active form folinic acid
- Essential for the formation of intercellular material, bone and teeth
- Essential for the absorption of iron[171]
- Fights bacterial and viral infections
- Helps in healing[172]
- Maintains healthy reproductive organs
- May help protect against certain cancers, cataracts and heart disease
- Necessary in production of red blood cells
- Prevents allergic reactions (antihistamine activity)
- Prevents hemorrhaging

**USES**

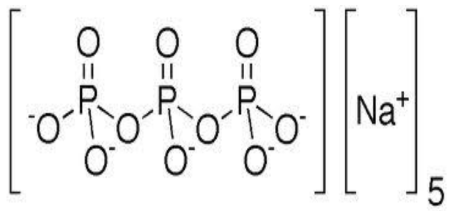
- Bone viral and bacterial infections
- Allergic reactions (due to its antihistamine qualities)
- Arthritis

- Atherosclerosis
- Colds and influenza
- Gastro-intestinal hemorrhages
- Iron-deficient anemia[173,174]
- Reducing high blood cholesterol levels
- Reducing leg cramps during pregnancy
- Treating lead, mercury and cadmium poisoning
- Treating loose teeth[167]
- Treatment of scurvy[175,176]
- Great antioxidant [175,176]
- Bleeding gums[167]
- Adjuvant in cancer[175]
- Reduces gastric cancer[177]
- Stabilizes food and plasma
- Coronary heart diseases[172]

## 7.2 SODIUM TRIPOLYPHOSPHATE

Sodium tri phosphate (STP, sometimes STPP or sodium tripolyphosphate or TPP,) is an inorganic compound with formula $Na_5P_3O_{10}$. It is the sodium salt of the polyphosphate penta-anion, which is the conjugate base of triphosphoric acid. It is produced on a large scale as a component of many domestic and industrial products, especially detergents[178].

**Chemical structure**



**Chemical and physical properties**

- White powder
- Melting point 622 degree centigrade
- Easily soluble in water
- Can soften hard water
- Slightly alkaline

**Specifications**

- **Molecular Formula :** $Na_5P_3O_{10}$
- **Molecular weight :** 367.86

**Uses**

- Manufacturing of ceramic tile
- Gum synthesis

- Food industry
- Poultry process
- Metal industry
- Pharmaceutical industry

**Storage**

- Avoid Moisture
- Should not be put in the open air

**Table 10 Details of sodium tripolyphosphate**

| **Indexes** | **Industrial Grade** | **Food Grade** |
|---|---|---|
| $Na_5P_3O_{10}$ | 94.0%Min | 94.0%Min |
| $P_2 O_5$ | 57.0%Min | 57.0%Min |
| Water Insoluble Matter | 0.15%Max | 0.04%Max |
| Iron(Fe) | 0.015%Max | 0.006%Max |
| PH value (1% water solution) | 9.2-10.0 | 9.5-10.0 |
| Heavy Metal (as Pb) | 70 PPM Max. | 0.001%Max |
| Arsenic (As) | - | 0.0003%Max |
| Fluoride (F) | - | 0.003%Max |
| Whiteness | 92%Min | 92%Min |
| Ignition loss | 1.0 | - |
| density (g/cm3) | 0.35-0.9 | 0.35-0.9 |

# 8. MATERIALS

**Table 11 List of materials**

| Drugs and Chemicals | | Supplier/ Manufacturer |
|---|---|---|
| Rifampicin | - | Astha laboratories Pvt Ltd, Hyderabad. |
| Isoniazid | - | Astha laboratories Pvt Ltd, Hyderabad. |
| Chitosan (85% deacetylation) | - | Central Institute of Fisheries Technology, |
| (M.W-150kDa, 400KDa, 600KDa) | - | Cochin. |
| Glacial acetic acid (Analytical grade) | - | S.D Fine chem. Ltd, Mumbai. |
| Ascorbic acid (Analytical grade) | - | Qualigens fine Chemicals, Mumbai. |
| Potassium chloride (Analytical grade) | - | Loba chemicals, Mumbai. |
| Hydrogen chloride (Analytical grade) | - | Loba chemicals, Mumbai. |
| HPLC water (Analytical grade) | - | Merck Pvt Ltd, Mumbai. |
| Sodium Hydroxide (Analytical grade) | - | Spectrum Reagents & Chemicals, Cochin. |
| Potassium Di hydrogen Phosphate (Analytical grade) | - | Spectrum Reagents & Chemicals, Cochin. |
| Tween 80(Analytical grade) | - | Loba chemicals, Mumbai. |
| Sodium Tri Poly Phosphate (Analytical grade) | - | S.D Fine chem. Ltd, Mumbai |
| Dialysis membrane (M.WCO 12,000-15,000Da) | - | Himedia Laboratory, Mumbai |

# 9. INSTRUMENTS

**Table 12 List of Instruments**

| Instruments | | Model / Manufacturer |
|---|---|---|
| FT-IR spectrophotometer | - | Perkin Elmer Spectrum RX 1, USA. |
| Scanning electron microscope | - | Joel model JSM 6400, Tokyo. |
| UV-Visible Spectrophotometer | - | Perkin Elmer Lambda 25, USA |
| Dissolution apparatus | - | Electro lab No.2 (USP XXIII) 1995, Chennai. |
| Single pan digital balance | - | Shimadzu BL220H , Koyoto, Japan. |
| Microscope | - | Unilab, Ambala cantt, Haryana, India. |
| Digital pH meter | - | Hanna instruments, Italy HI98. |
| Magnetic stirrer | - | Eltek MS 2012, India. |
| Sonicator | - | Bandelin Sono plus Model HD, 2070. |
| Freeze Drier | - | Labconico, USA |
| Research cooling centrifuge | - | Remi Centrifuge R4C-DX, USA |
| Differential Scanning Colorimetry | - | DSC CA 60 Shimadzu, Japan |
| Zeta potential analyzer | - | Zetasizer 3000HS, Malvern instrument, UK. |

# 10. METHODS

**Estimation of pure rifampicin**

Rifampicin was estimated spectrophotometrically at 475 nm [179,180,181,182,183] in the range of 2-10 µg/ml as per Beer Lambert's law[184].

**Preparation of buffer pH 1.2**

50ml 0f 0.2M KCl was mixed with 85ml of 0.2M HCl and made upto 200ml with water.

**Preparation of 0.2M potassium chloride**

14.911gm of KCl was dissolved in distilled water and diluted with distilled water and made up to 1000ml[185]

**Preparation of 0.2M hydrochloric acid**

17ml of HCl was mixed with 1000ml 0f $H_2O$[185].

**10.1 Preparation of standard graphs**

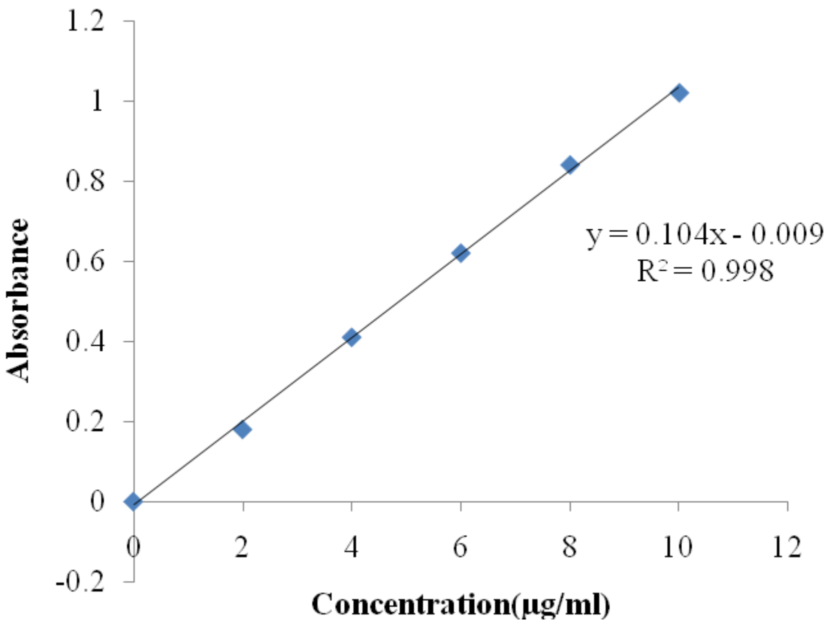
**Preparation of standard solution**

100mg of rifampicin was dissolved in 100ml of buffer pH 1.2 so as to get a stock solution of 1000 µg/ml concentration. From this 2 ml of stock solution was diluted to 100ml with pH 1.2 phosphate buffer thus giving a concentration of 20 µg/ml of the drug. Aliquot of standard drug solution ranging from 1ml to 9ml were transferred to 10ml volumetric flask and were diluted up to the mark with pH 1.2 buffer. Thus the final concentration ranges from 2-10µg/ml as per Beer Lambert'slaw[184]. Absorbance of each solution was measured at 475nm[179,180,181,182,183] against buffer pH 1.2 as a blank and the concentrations of drug vs absorbance were plotted.

**Table 13 Absorbance of rifampicin at 475nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.18 |
| 3. | 4 | 0.41 |
| 4. | 6 | 0.62 |
| 5. | 8 | 0.84 |
| 6. | 10 | 1.02 |

**Figure 4. Standard curve of rifampicin at pH 1.2 buffer**



**Preparation of standard graph of rifampicin with ascorbic acid (125, 250, 500, 1000mg)**

**Preparation of standard solution**

100mg of rifampicin and ascorbic acid (125, 250,500, 1000mg) were dissolved in 100ml of buffer pH 1.2 so as to get a stock solution of 1000 μg/ml concentration. From this 2 ml of stock solution was diluted to 100ml with pH 1.2 phosphate buffer thus giving a concentration of 20 μg/ml of the drug. Aliquot of standard drug solution ranging from 1 to 9ml were transferred to 10ml volumetric flask and were diluted up to the mark with pH 1.2 buffer. Thus the final concentration ranges from 2-10 μg/ml as per Beer Lambert's law. Absorbance of each solution was measured at 475 nm against buffer pH 1.2 as a blank and the concentrations of drug vs absorbance were plotted.

**Table 14 Absorbance of rifampicin with ascorbic acid (125mg) at 475nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.21 |
| 3. | 4 | 0.42 |
| 4. | 6 | 0.64 |
| 5. | 8 | 0.86 |
| 6. | 10 | 0.99 |

**Figure 5. Standard curve of rifampicin with ascorbic acid (125mg) at pH 1.2 buffer**

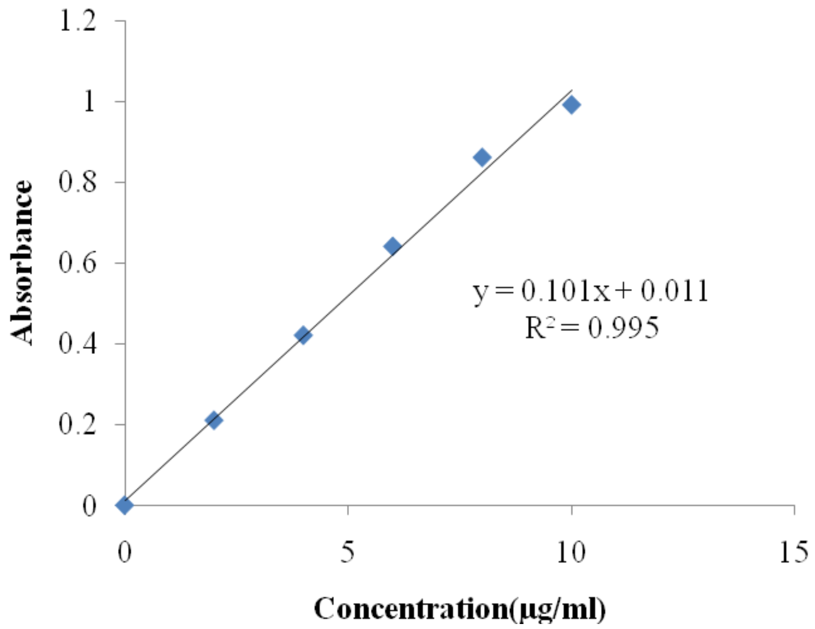


**Table 15 Absorbance of rifampicin with ascorbic acid (250mg) at 475nm in pH 1.2 buffer**

| S.No | Concentration(µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.23 |
| 3. | 4 | 0.40 |
| 4. | 6 | 0.59 |
| 5. | 8 | 0.74 |
| 6. | 10 | 0.92 |

**Figure 6. Standard curve of rifampicin with ascorbic acid (250mg) at pH 1.2 buffer**

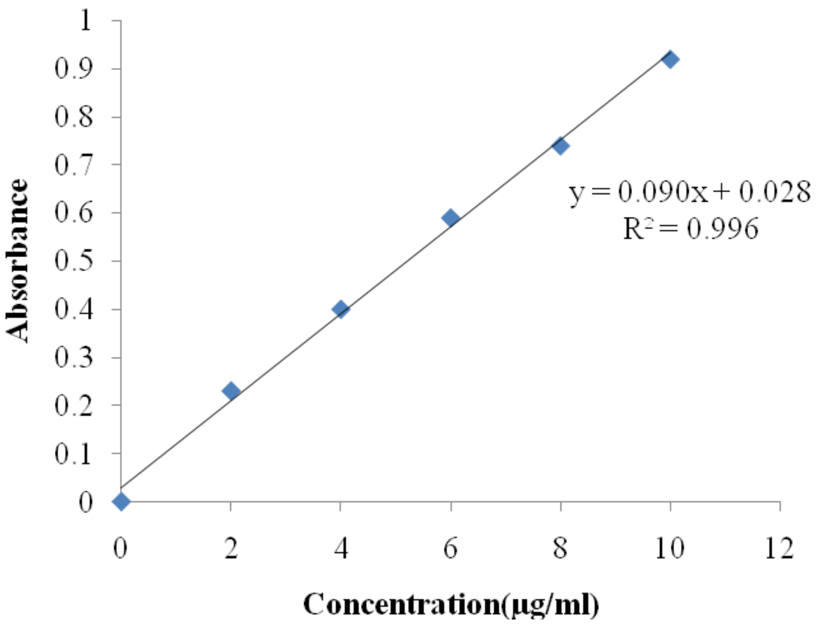


**Table 16 Absorbance of rifampicin with ascorbic acid (500mg) at 475nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.21 |
| 3. | 4 | 0.41 |
| 4. | 6 | 0.61 |
| 5. | 8 | 0.82 |
| 6. | 10 | 0.98 |

**Figure 7. Standard curve of rifimpicin with ascorbic acid (500mg) at pH 1.2 buffer**

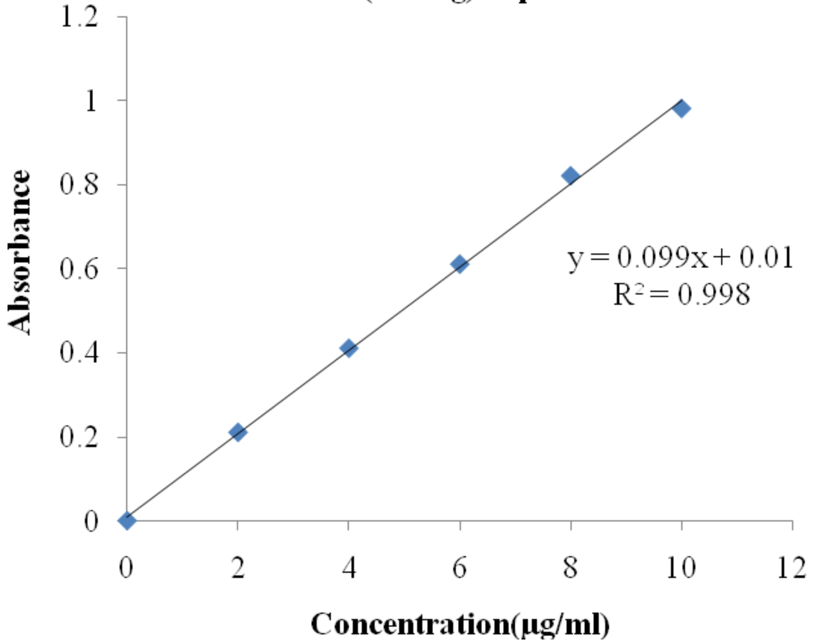
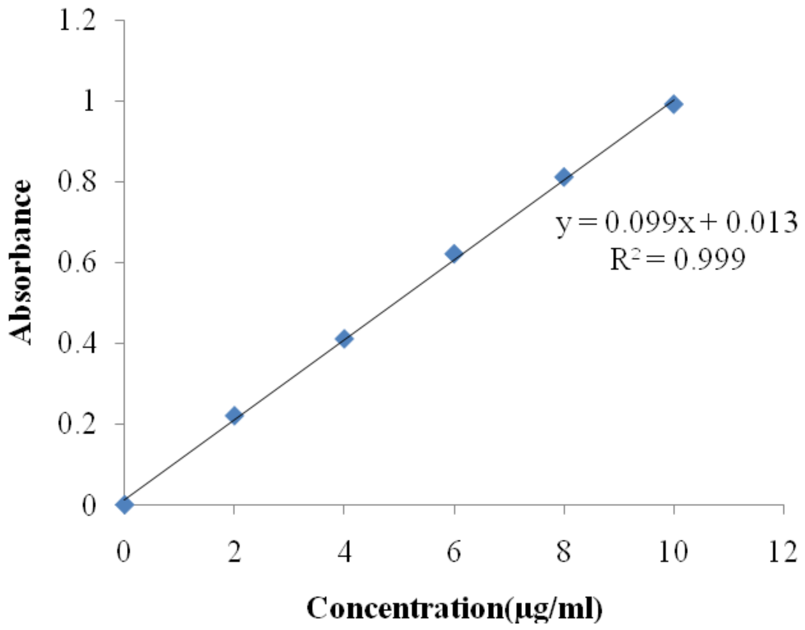


**Table 17 Absorbance of rifampicin with ascorbic acid (1000 mg) at 475nm in pH 1.2 buffer**

| S.No | Concentration(μg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.22 |
| 3. | 4 | 0.41 |
| 4. | 6 | 0.62 |
| 5. | 8 | 0.81 |
| 6. | 10 | 0.99 |

**Figure 8. Standard curve of rifimpicin with ascorbic acid (1000mg) at pH 1.2 buffer**

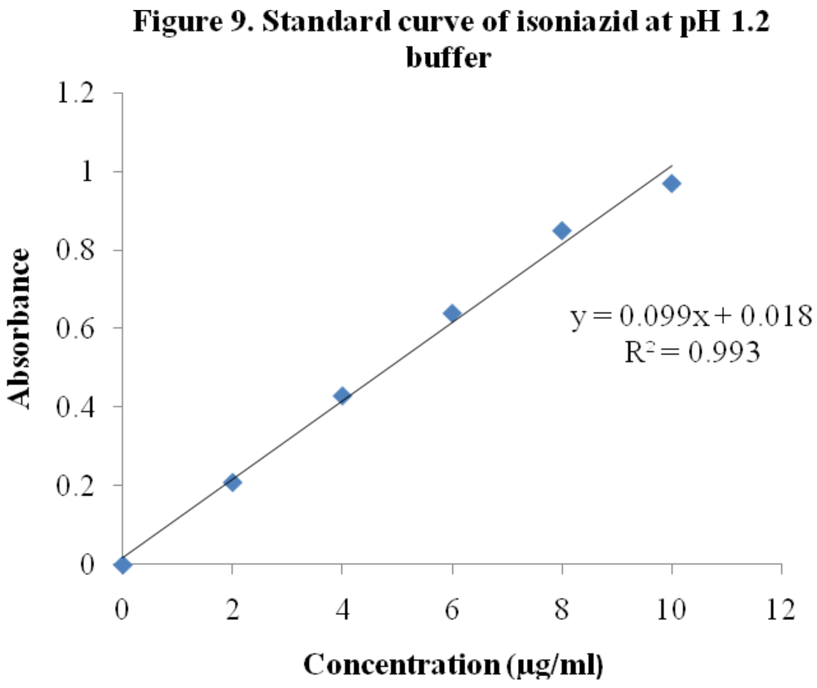


**Preparation of standard graph for isoniazid**

100mg of isoniazid was dissolved in 100ml of buffer pH 1.2 so as to get a stock solution of 1000 μg/ml concentration. From this 2 ml of stock solution was diluted to 100ml with pH 1.2 phosphate buffer thus giving a concentration of 20 μg/ml of the drug. Aliquot of standard drug solution ranging from 1ml to 9ml were transferred to 10ml volumetric flask and were diluted up to the mark with pH 1.2 buffer. Thus the final concentration ranges from 2-10 μg/ml as per Beer Lambert's law. Absorbance of each solution was measured at 263 nm[186] against buffer pH 1.2 as a blank and the concentrations of drug vs absorbance were plotted.

**Table 18 Absorbance of isoniazid at 263nm in pH 1.2 buffer**

| S.No | Concentration(µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.21 |
| 3. | 4 | 0.43 |
| 4. | 6 | 0.64 |
| 5. | 8 | 0.85 |
| 6. | 10 | 0.97 |



**Procedure for simultaneous estimation of rifampicin and isoniazid**

Procedure for determining the sampling wavelength for simultaneous analysis.

By appropriate dilution of two standard drug solutions with ethanol, solutions containing 10 µg/ ml of rifampicin and 10 µg/ml of isoniazid were scanned separately in the range of 500-200 nm to determine the wavelength of rifampicin and isoniazid showed absorbance maxima at 475[179,180,181,182,183] and 263[186] nm respectively as shown in Fig. 8 and 9.

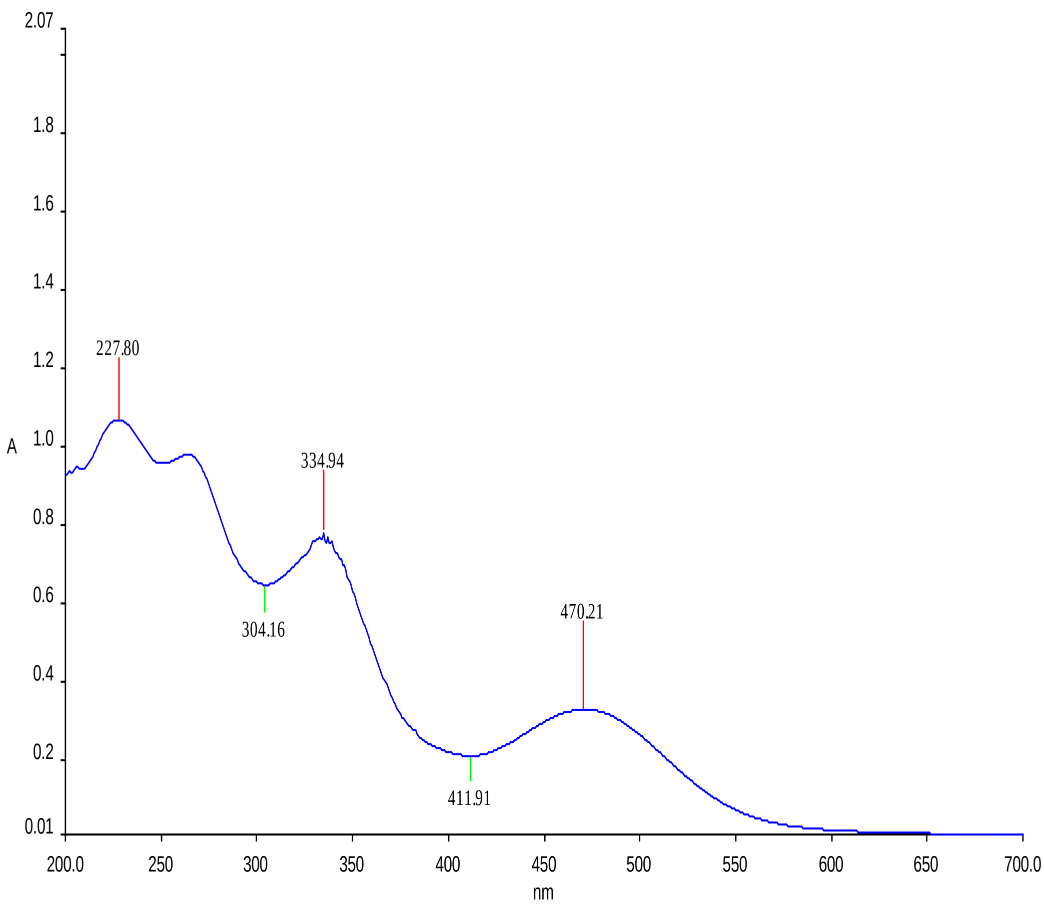



**Figure 10. Absorption spectrum of rifampicin in buffer pH 1.2 (10 µg/ml) showing λ max 475nm**

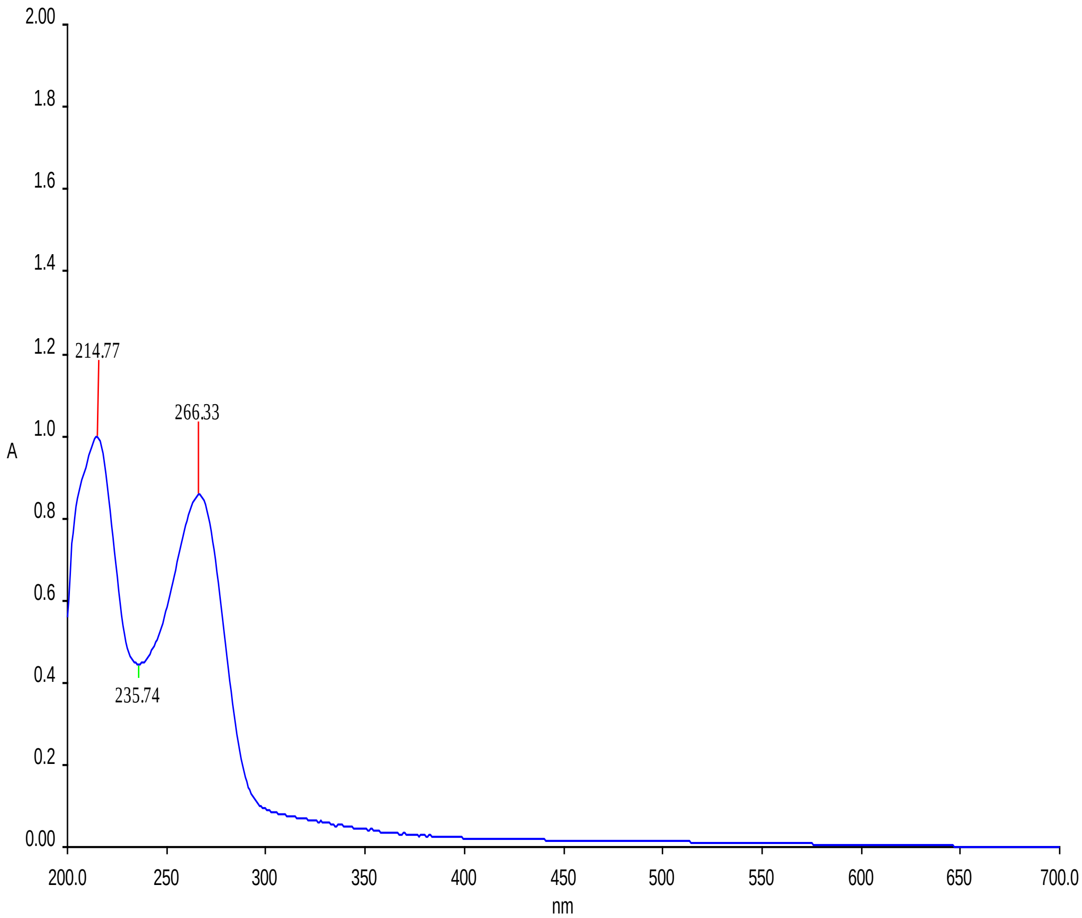



**Figure 11. Absorption spectrum of rifampicin in buffer pH 1.2 (10 µg/ml) showing λ max 263nm**

**Selection of method and wavelength**

For estimation of rifampicin, simultaneous equation method employing 475 nm as analytical wavelength was used. For estimation of isoniazid, 263 nm was selected as the analytical wavelength. In the simultaneous equation method developed for simultaneous estimation of rifampicin and isoniazid, the wavelengths were selected from the overlain spectra as shown in Fig 10.

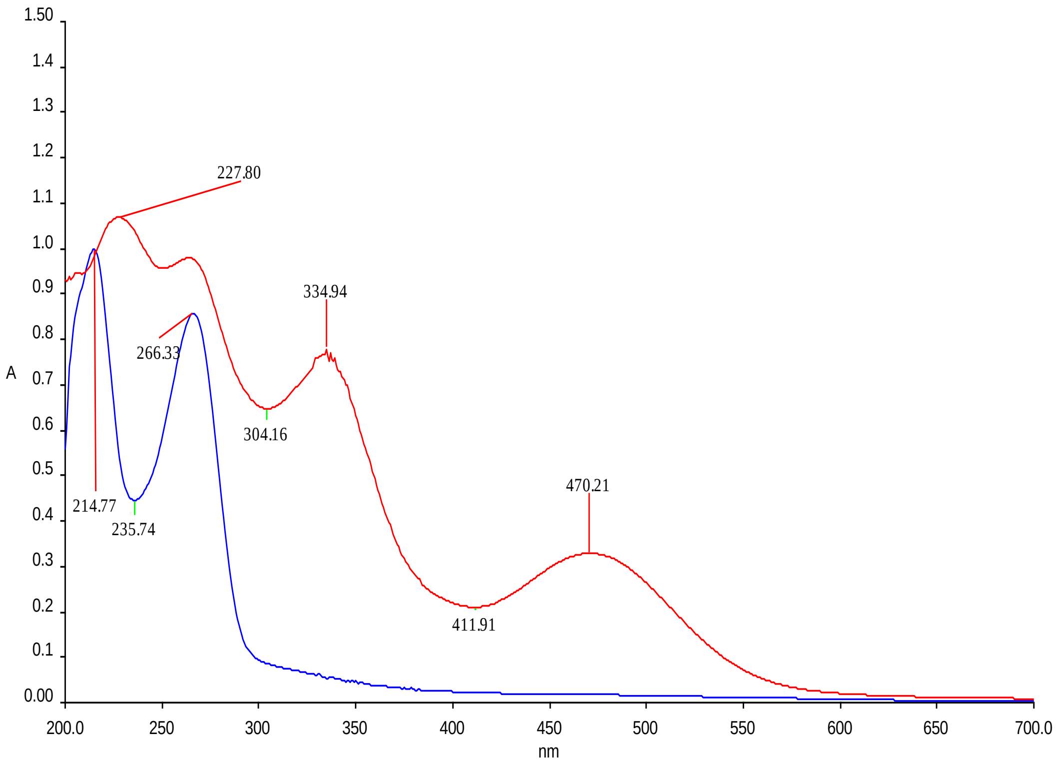


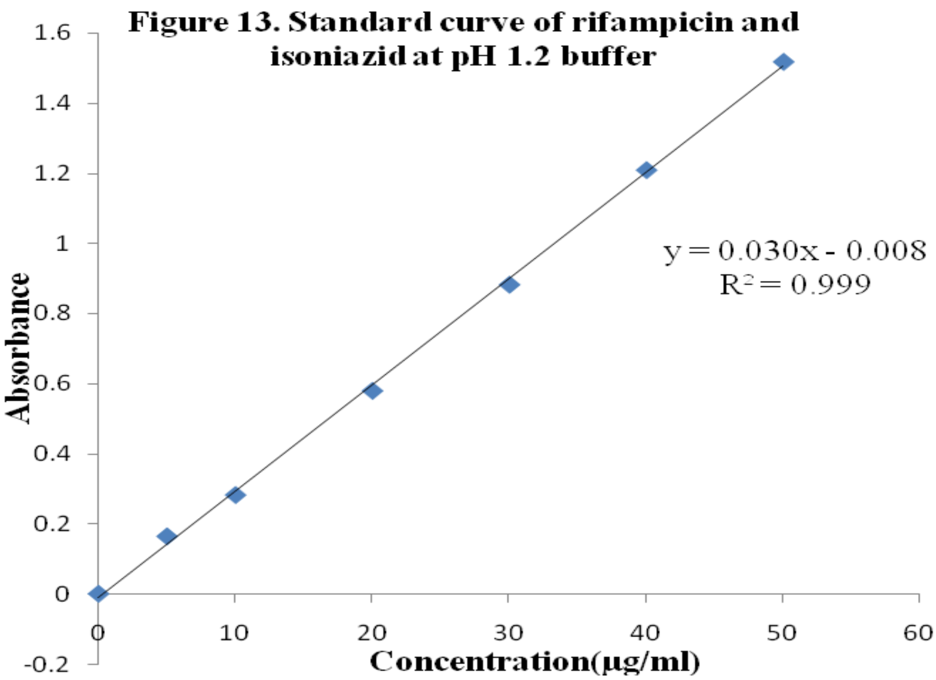

**Figure 12. Overlain spectra of isoniazid and rifampicin in buffer pH 1.2 (10 µg/ml) showing $\lambda_{max}$ 263nm and 475nm in standard solution**

**Preparation of Standard Stock Solution**

The standard stock solution containing rifampicin and isoniazid were prepared by dissolving 100 mg of rifampicin and 100mg of isoniazid separately in 20 ml of buffer pH 1.2. It was then sonicated for 10 minutes and the final volume of both the solutions were made up to 100 ml with buffer pH 1.2 to get stock solutions containing 1000 µg/ ml each of rifampicin and isoniazid in two different 100 ml volumetric flasks. From this 2 ml of stock solution was diluted to 100ml with pH 1.2 phosphate buffer thus giving a concentration of 20 µg/ml of the drug. Aliquot of standard drug solution ranging from 1 to 9ml were transferred to 10ml volumetric flask and were diluted up to the mark with pH 1.2 buffer. Thus the final concentration ranges from 2-10 µg/ml as per Beer Lambert's law. Absorbance of each solution was measured at 215 nm against buffer pH 1.2 as a blank and the concentrations of drug vs absorbance were plotted.

**Table 19 Absorbance of rifampicin and isoniazid at 215nm in pH 1.2 buffer**

| S.No | Concentration(µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.164 |
| 3. | 4 | 0.282 |
| 4. | 6 | 0.579 |
| 5. | 8 | 0.882 |
| 6. | 10 | 1.209 |



**Preparation of standard graph for rifampicin and isoniazid with ascorbic acid (125, 250,500, 1000mg)**

100mg of rifampicin, 100mg of isoniazid, and ascorbic acid (125, 250,500, 1000mg**)** were dissolved in 100ml of buffer pH 1.2 so as to get a stock solution of 1000 µg/ml concentration. From this 2 ml of stock solution was diluted to 100ml with pH 1.2 phosphate buffer thus giving a concentration of 20 µg/ml of the drug. Aliquot of standard drug solution ranging from 1ml to 9ml were transferred to 10ml volumetric flask and were diluted up to the mark with pH 1.2 buffer. Thus the final concentration ranges from 2-10 µg/ml as per Beer Lambert's law. Absorbance of each solution was measured at 215 nm against buffer pH 1.2 as a blank and the concentrations of drug vs absorbance were plotted.

**Table 20 Absorbance of rifampicin and isoniazid with ascorbic acid (125 mg) at 215nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.17 |
| 3. | 4 | 0.37 |
| 4. | 6 | 0.56 |
| 5. | 8 | 0.74 |
| 6. | 10 | 0.90 |

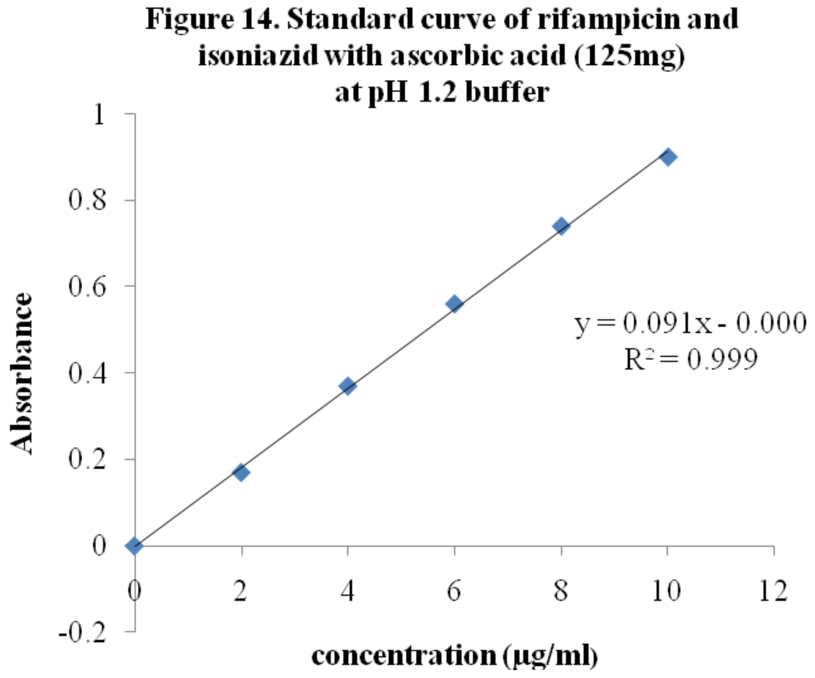


**Table 21 Absorbance of rifampicin and isoniazid with ascorbic acid (250 mg) at 215nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.27 |
| 3. | 4 | 0.43 |
| 4. | 6 | 0.63 |
| 5. | 8 | 0.84 |
| 6. | 10 | 0.99 |

**Figure 15. Standard curve of rifampicin and isoniazid with ascorbic acid (250mg) at pH 1.2 buffer**

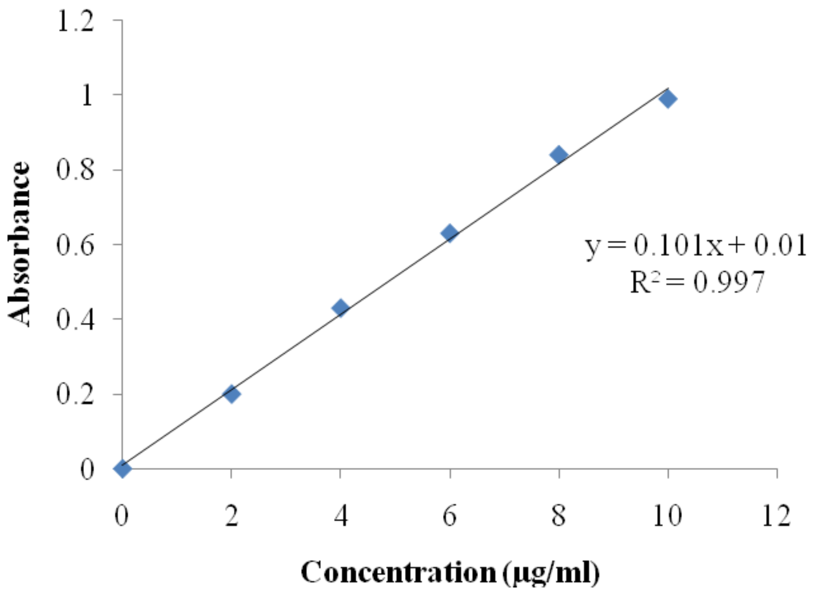


**Table 22 Absorbance of rifampicin and isoniazid with ascorbic acid (500mg) at 215nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.20 |
| 3. | 4 | 0.38 |
| 4. | 6 | 0.67 |
| 5. | 8 | 0.73 |
| 6. | 10 | 0.91 |

**Figure 16. Standard curve of rifampicin and isoniazid with ascorbic acid (500mg) at pH 1.2 buffer**

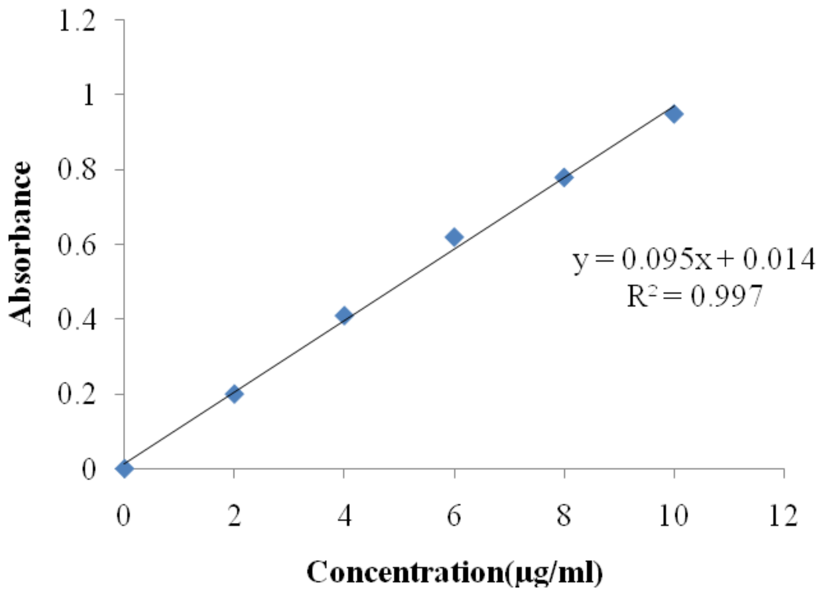
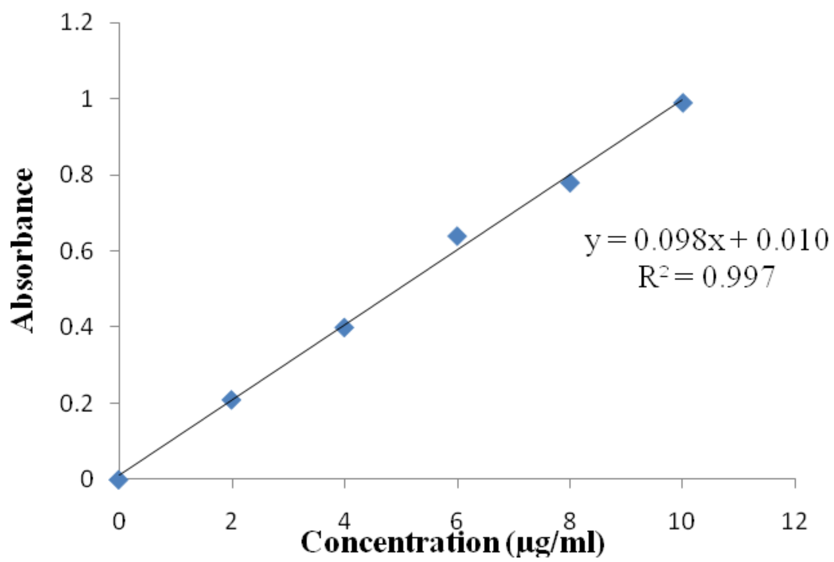


**Table 23 Absorbance of rifampicin and isoniazid with ascorbic acid (1000mg) at 215nm in pH 1.2 buffer**

| S.No | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 2 | 0.2 |
| 3. | 4 | 0.41 |
| 4. | 6 | 0.62 |
| 5. | 8 | 0.78 |
| 6. | 10 | 0.95 |

**Figure 17. Standard curve of rifampicin and isoniazid with ascorbic acid (1000mg) at pH 1.2 buffer**



### 10.2 Preparation of rifampicin loaded chitosan nanoparticles

Rifampicin loaded chitosan nanoparticles were prepared according to the procedure first reported by Calvo et al (1997b)[187] based on the ionic gelation of chitosan with sodium tri polyphosphate anions (STPP) in the presence of tween 80 as a suspending agent to prevent aggregation, at ambient temperature. Rifampicin and chitosan with different molecular weight (150, 300, 450KDa) were dissolved in 1% acetic acid in aqueous solution under magnetic stirring at room temperature in the presence of tween 80 at various concentrations (0.25, 0.5, 1.0%). Nanoparticles were prepared by adding STPP aqueous solution drop wise to chitosan solution under magnetic stirring with different speed (400,800,1200rpm) at room temperature for 45 min. The nanosuspensions were cold centrifuged at 12000g, in a glucose bed for 30 min using Remi centrifuge (R4C-DX, USA). The supernatant liquid was analyzed by spectrophotometer at 475 nm[179,180,181,182,183] to calculate the percentage drug entrapment and drug loading. The final suspensions were then frozen and lyophilized at 0.4 mbar and -40 ºC for 5 hrs using sucrose and glucose (1:1) as cryoprotective agents. The lyophilized nanoparticles were stored in desiccators at 4ºC. The formula for preparation of nanoparticles is given in Table 23.

### 10.3 Preparation of rifampicin-ascorbic acid nanoparticles

The chitosan molecular weight and ascorbic acid concentration were chosen based on maximum diffusion and dissolution stability characteristics respectively for the preparation of rifampicin-ascorbic acid loaded chitosan nanoparticles. The nanoparticles were prepared as described before under preparation of nanoparticles (10.2).

**Table 24 Formulation of rifampicin loaded chitosan nanoparticles**

| Formula code | Composition | | |
|---|---|---|---|
| | **Chitosan Mwt (KDa)** | **(%) Tween 80** | **Stirring speed(rpm)** |
| F1 | 150 (Low) | 0.25 | 400 |
| F2 | | 0.5 | 400 |
| F3 | | 1.0 | 400 |
| F4 | | 0.25 | 800 |
| F5 | | 0.5 | 800 |
| F6 | | 1.0 | 800 |
| F7 | | 0.25 | 1200 |
| **F8** | | **0.5** | **1200** |
| F9 | | 1.0 | 1200 |
| F10 | 300 (Medium) | 0.25 | 400 |
| F11 | | 0.5 | 400 |
| F12 | | 1.0 | 400 |
| F13 | | 0.25 | 800 |
| F14 | | 0.5 | 800 |
| F15 | | 1.0 | 800 |
| F16 | | 0.25 | 1200 |
| F17 | | 0.5 | 1200 |
| F18 | | 1.0 | 1200 |
| F19 | 600 (High) | 0.25 | 400 |
| F20 | | 0.5 | 400 |
| F21 | | 1.0 | 400 |
| F22 | | 0.25 | 800 |
| F23 | | 0.5 | 800 |
| F24 | | 1.0 | 800 |
| F25 | | 0.25 | 1200 |
| F26 | | 0.5 | 1200 |
| F27<br>**F28** | **150 (Low)** | 1.0<br>**0.5** | 1200<br>**1200** |

## 10.4 Evaluation of rifampicin loaded chitosan nanoparticles

### 10.4.1 Drug and carrier interaction by Fourier transform infra red spectroscopy (FTIR)

FTIR Spectroscopy (Perkin Elmer RX1) was performed on pure drug, polymer and nanoparticles. The pellets were prepared by gently mixing 1mg sample with 200mg potassium bromide at high compaction pressure. The scanning range was 450 to 4000 $cm^{-1}$ and the resolution was 4 $cm^{-1}$. The pellets thus prepared were examined and the spectra of all the samples were compared.

### 10.4.2 Thermal analysis by differential scanning calorimetry (DSC)

Differential scanning calorimetric measurement of nanoparticles was carried out by using a thermal analysis instrument (DSC CA 60 Shimadzu. Japan) equipped with liquid nitrogen sub ambient accessory. Samples were accurately weighed in aluminum pans thematically sealed and heated at a rate of 10ºC $min^{-1}$ in a 30 to 300ºC temperature under nitrogen flow of 40 ml/min. The experiment was repeated for pure rifampicin, chitosan, ascorbic acid and physical mixture.

## 10.5 Physicochemical properties and release of nanoparticles

### 10.5.1 Morphology by scanning electron microscopy

The morphology of nanoparticles was analyzed by scanning electron microscope (JEOL MODEL JSM 6400). The nanoparticles were mounted directly on the SEM stub, using double –sided, sticking tape and coated with platinum and scanned in a high vacuum chamber with a focused electron beam. Secondary electrons, emitted from the samples were detected and the image formed.

### 10.5.2 Surface characteristics by zetasizer

The particle size, and particle size distribution of nanoparticles were measured with a malvern instrument (Zetasizer 3000 HS U.K).The particle size distribution is reported as poly dispersity index. The samples were placed in the analyzer chamber and readings were performed at 25°c with a detected angle of 90 degrees. The zeta potential of nanoparticles was measured with a malvern instrument (Zetasizer 3000 HS U.K). The samples were diluted with pH 7.4 buffer, and placed in eletrophoretic cell and measured in the automatic mode.

### 10.5.3 Rifampicin encapsulation efficiency and loading capacity of nanoparticles

The encapsulation efficiency and loading capacity of nanoparticles were determined by the separation of nanoparticles from the supernatant liquid containing non associated rifampicin obtained after cold centrifugation at 12000g for 30 minutes .The amount of free rifampicin in the supernatant liquid was measured by spectrophotometer at 475 nm. The rifampicin encapsulation efficiency (EE) and loading capacity (LC) of the nanoparticles were calculated from the following equations[188].

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of rifampicin} - \text{Free rifampicin}}{\text{Total amount of rifampicin}} \times 100$$

$$\text{Loading capacity} = \frac{\text{Total amount of rifampicin} - \text{Free rifampicin}}{\text{Weight of nanoparticles}} \times 100$$

## 10.6 *In-vitro* dissolution stability study

*In-vitro* dissolution stability was performed on rifampicin, rifampicin nanoparticles and rifampicin – ascorbic acid nanoparticles in 0.1 N HCL. The study was carried out by the method described by Shisoo et al., (1999)[19]. A solution of 0.1N HCL (200 ml) was placed in the vessel of the USP dissolution apparatus No.2 (USP XXIII, 1995) and the medium was equilibrated at 37±0.1°C with stirring at 50 rpm. Rifampicin (100mg) and ascorbic acid (drug to ascorbic acid ratio at 100:125, 100:250, 100:500,100:1000mg) were accurately weighed, dissolved in and diluted to 100 ml with 0.1N HCL. The resulting solution was transferred immediately to the dissolution vessel at once and 1 ml of sample was withdrawn immediately from a zone midway between the surface of the dissolution medium and bottom of the vessel (0-min sample). This experiment was done in the presence and absence of isoniazid in a concentration proportionate to the strength in FDC products taken in the medium. Samples were withdrawn at 0, 15, 30, 45, 60min intervals and filtered through 0.1µm membrane filter immediately and 1 ml fresh 0.1N HCL solution was added in to the system. An aliquot, 1 ml was diluted to 10 ml of 0.1N HCL using cyclomixer (3min). Samples were measured at 475nm in spectrophotometer[179,180,181,182,183]. The experiment was run in triplicate and the mean values were recorded as percent drug degradation. The percentage degradation of drug was calculated from the difference between the values of sample drawn at 0 and appropriate time intervals[69].

**10.7 *In-vitro* diffusion study**

The studies were performed on rifampicin, rifampicin nanoparticles and rifampicin – ascorbic acid nanoparticles in 0.1 N HCL. Sample equivalent to 100mg of rifampicin was redispersed in 10ml 0.1N HCL solution and placed in a dialysis membrane bag with a molecular cut-off of (MWCO 12,000-15,000Da, Himedia, India) which acts as a donor compartment, tied and placed into 10 ml 0.1N HCL solution in a beaker which acts as a receptor compartment .The entire system was kept at 37ºC±0.1°C with continuous magnetic stirring at a rotation speed of 50 rpm. At appropriate time intervals (0, 15, 30, 45, 60min) 1 ml of the release medium was removed through 0.1µm membrane filtered immediately and 1 ml fresh 0.1N HCL solution was added in to the system. This experiment was done in the presence and absence of isoniazid taken in the medium. The amount of rifampicin in the release medium was determined by spectrophotometer at 475nm and the percentage release of rifampicin recorded. The experiment was run in triplicate and the mean values were recorded as percent release of rifampicin[189].

**10.8 Pharmacokinetics study**

**Animals**

New Zealand white rabbits weighing 1.5 to 2.5kg were obtained from the animal house, Swamy Vivekananda College of Pharmacy. The animals were fed with cabbage and water. They were maintained in standard laboratory conditions at 21±2 $^0$C and relative humidity of 55-60%. The animals are overnight fasted before the experiment. The study protocol was approved by the Institutional Animal Ethical Committee and the protocol number is SVCP/IAEC/Ph.D /03/2011.

**Drugs:** Rifampicin and isoniazid were obtained from **Astha Laboratories Pvt. Ltd,** Hyderabad, Andhra Pradesh, India. Ascorbic acid was obtained from Qualigens Fine Chemicals, Mumbai.

**Chemicals:** 1% CMC from Loba Chem Pvt Ltd, Mumbai, 95% v/v alcohol.

**Requirements:** Cotton, Surgical blade, 26G needle, Blood collecting tubes (EDTA tubes), Plasma sample collecting tubes

**Sex:** Both Male/Female

**No. of animals: 24**

**Animal dose:**

Rifampicin : 31mg/kg/p.o

Isoniazid : 15mg/kg/p.o

Ascorbic acid : 26mg/kg/p.o

**Collection of blood from marginal ear vein**

The animal was placed in a retainer. Hair of the ear was shaved smoothly with blade without disturbing the blood vessels. Ear was cleaned with 95% v/v alcohol on the collection site and rapid rubbing on the ear to dilate blood vessels which is easy to collect the blood. 2G needle was inserted in vein to collect the blood from marginal ear vein. After collecting blood, clean sterile cotton was kept on the collection site and finger pressure was applied to stop the bleeding[190].

Rabbits were classified into 8 different groups each group consisting of 3 animals.

Group I - Control (Rifampicin)

Group II - Rifampicin + Isoniazid

Group III - Rifampicin - ascorbic acid

Group IV - Rifampicin + Isoniazid - ascorbic acid

Group V - Rifampicin nanoparticles

Group VI - Rifampicin nanoparticles + Isoniazid

Group VII - Rifampicin - ascorbic acid nanoparticles

Group VIII - Rifampicin - ascorbic acid nanoparticles + Isoniazid

**Procedure**

**Preparation of samples**

The samples were suspended in water using 1% CMC as a suspending agent and used for this study. Each group of animals (three rabbits) was administered the samples as shown above through an intra gastric tube after a overnight fasting.

Blood samples (1 ml) were collected in to heparinized tubes from the marginal ear vein at 0, 0.5, 1, 2, 4, 6, 9 and 12 h after drug administration and plasma was separated by using centrifugation and stored at -20°C. Samples were analyzed by high performance liquid chromatography (HPLC) [191].

**Bioanalytical work**

**Extraction of rifampicin from plasma**

The method described by Rafig *et al.,* (2010) [192] was followed. The mobile phase consisted of acetonitrile, 0.05 M sodium citrate buffer adjusted to pH 4.0 with 0.05 M hydrochloric acid (42:58) and pumped at a flow-rate of 2.3 ml/min an ambient temperature. Buffer was filtered through Whatman filter paper. Mobile phase was filtered (cellulose acetate filter diameter 47 mm, pore size 0.45 µm, Sartorius AG. 370700) and sonicated (EYELA-Sonicator) for 12 min.

An aliquot of 200 µl of plasma samples was pipetted into an eppendrof's tube of 1.5ml capacity. Acetonitrile (300 µl) was added, vortexed for one minute and micro centrifuged at 10,000 rpm for 5min. Then 300 µl of the supernatant was taken into another micro centrifuge tube and vacuum dried in the HETO vacuum centrifuge. The residue obtained was reconstituted in 100 µl of mobile phase. Plasma was filtered through 0.22 µm membrane (13 mm) and 20 µl volumes were injected[193].

**HPLC analysis**

Rifampicin was analyzed at ambient temperature on a Kromasil C18 column Phenomenex, UK, 100mm×3.2mm i.d., 3µm particle size) with an ODS Securigard® guard column 4mm×3mm (Phenomenex, Macclesfield, UK). The mobile phase ammonium acetate buffer was adjusted to pH 4.0 with 0.05 M hydrochloric acid and acetonitrile (20:90). The analytes were detected by a UV –

Visible detector at 475 nm[194]. The injection volume for each sample was 20µl to column. The peak was found at 2.52 min, which was probably the 25-desacetylrifampicin metabolite. HPLC analyses showed that the assay method is linear in the ranges 0.1-1µg/ml for plasma. The chromatograms were recorded according to the "Area normalization method" with the measurement of peak area[195].

**Extraction efficiency**

The extraction efficiency was calculated by comparing the peak heights of rifampicin spiked-pooled blank plasma samples with that of respective standard rifampicin samples.

**Calibration curve for rifampicin**

A calibration curve of rifampicin was obtained by plotting the concentration of rifampicin against the respective spiked-pooled blank plasma samples peak area. Concentration of rifampicin was calculated by using the following equation.

$Y = MX + C$

Peak plasma concentration ($C_{max}$) and the time to attain the peak plasma concentration ($T_{max}$) were calculated from the actual plasma data. The elimination rate constant ($K_{el}$) was calculated from the log-linear decline of concentration. Elimination half life ($t_{1/2}$) was obtained from $t_{1/2}$ el=0.693/ $K_{el}$. Absorption rate constant ($k_a$) was calculated by the residual method and absorption half life ($t_{1/2}$ a) was obtained from the formula $t_{1/2}$ a=0.693/$K_a$. Area under the plasma drug concentration versus time curve (AUC $_{0-12}$ h) was calculated by trapezoidal rule. Extension of the AUC data to infinity (AUC $_{0-\infty}$) was done by dividing the last observed concentration of drug in plasma by $K_{el}$[196].

**Statistical analysis**

The data were analyzed by one way ANOVA followed by Tukey's multiple comparison tests with the help of Graph Pad Instat software, version 3.01. All the data were presented as a mean value with its standard deviation (mean±S.D). $P<0.05$ was considered as statistically significant.

# 11. RESULTS AND DISCUSSION

## 11.1 Preparation of rifampicin nanoparticles

The present study followed ionic gelation method for preparation of rifampicin nanoparticles using the natural and biodegradable polymer chitosan of different molecular weights. Natural polymers are more desirable for nanoparticles used in chronic infections like tuberculosis as they are free from polymer induced toxicity[28] and therefore chitosan was chosen in the study. Ionic gelation is simple to process and nanoparticles of desirable size are easily obtained. Chitosan with different molecular weight, tween 80 in variable concentrations and variable stirring speed were used in order to examine their influence on the physicochemical and release characteristics of the drug. Lyophillization was followed for preparation of nanoparticles using sucrose and glucose (1:1) as cryoprotective agent to yield stable amorphous solid with the desired property such as high redispersion speed, acceptable storage stability and also residual moisture content. Formulation of rifampicin loaded chitosan nanoparticles are given in Table 24.

## 11.2 Drug and carrier interaction by Fourier Transform Infra Red Spectroscopy

The FTIR spectra of drug, polymer, ascorbic acid and nanoparticles are shown in Figures 18, 19. The infrared spectra are recorded on Fourier Transform Spectrometer in the mid–infrared region (MIR) within the range (400-4000 $cm^{-1}$) and the study was carried out separately to check the compatability between drug and polymer used for the preparation of nanoparticles. There are three characteristic peaks of chitosan of different grades; 3318 $cm^{-1}$ of $\upsilon$(OH),1078$cm^{-1}$ of $\upsilon$(C-O-C) and 1621$cm^{-1}$ of $\upsilon(NH_2)$ for low molecular weight chitosan; 3267 $cm^{-1}$ of $\upsilon$(OH),1078$cm^{-1}$ of $\upsilon$(C-O-C) and 1675$cm^{-1}$ of $\upsilon(NH_2)$ for medium molecular weight chitosan;3324 $cm^{-1}$ of $\upsilon$(OH),1317$cm^{-1}$ of $\upsilon$(C-O-C) and 1578$cm^{-1}$ of $\upsilon(NH_2)$ for high molecular weight chitosan; The peaks of chitosan at 1621$cm^{-1}$ (low molecular weight), and at 1675$cm^{-1}$ (medium molecular weight), 1578$cm^{-1}$ (high molecular weight) disappeared and new sharp peaks at 1664 $cm^{-1}$, 1421$cm^{-1}$,1378 $cm^{-1}$ were observed in the chitosan-TPP nanoparticles. These changes indicate that amino groups of chitosan were cross linked with tripolyphosphate groups of TPP in the nanoparticles[197]. Rifampicin showed characteristic peaks at 1719$cm^{-1}$ (acetoxyl C=O), 1746$cm^{-1}$ (furanone C=O) and 2898$cm^{-1}$(amyl NH-C=O) and ascorbic acid showed characteristic peaks at 1012$cm^{-1}$ ; O-H, 1750$cm^{-1}$ ; C=O and at 1640$cm^{-1}$; C=C and these peaks were also observed in the rifampicin, rifampicin-ascorbic acid loaded chitosan nanoparticles (Figures.18h, 18i, 18j, 18k) indicating the absence of chemical interaction between the drug and polymer and thus safe use of ionic gelation method for development of rifampicin nanoparticles using chitosan as polymer.

**11.3 Thermal analysis by differential scanning calorimetry (DSC)**

The thermogram of rifampicin, chitosan, chitosan-TPP nanoparticles, rifampicin-chitosan nanoparticles are shown in Figures 20, 21. DSC analysis reveals the physical state of drug in the formulation which influences the release characteristics of the drug from the system. The drug and the polymer may co-exist in the polymeric carrier as a) amorphous drug either in amorphous or in crystalline polymer or b) crystalline drug either in amorphous or in crystalline polymer. The DSC thermogram of rifampicin showed endothermic peaks at 189.0$^o$C, 238.16$^o$C and exothermic peak at 272.02$^o$C (Figure.20a). The DSC of polymer showed characteristic endothermic and exothermic peaks at 101.42$^o$C and 290.03$^o$C for low molecular weight (Fig.20b), at 178.18$^o$C and 312.04$^o$C for medium molecular weight (Figure.20c), and at 182.49$^o$C and 338.4$^o$C for high molecular weight (Fig.20d) chitosan. The endothermic and exothermic peaks of chitosan of 101.42$^o$C and 290.03$^o$C (low Mwt), 178.18$^o$C and 312.04$^o$C (medium Mwt), 182.49$^o$C and 338.4$^o$C (high Mwt) were shifted to 113.72$^o$C and 310.12$^o$C, 180.98$^o$C and 320.1$^o$C, 182.49 and 340.2$^o$C respectively in chitosan-TPP nanoparticles (Figures. 20 e, f, g). Similarly the endothermic peaks of rifampicin at 189.0 $^o$C and 238.16$^o$C and 272.02$^o$C were shifted to 124.32 and 325.16$^o$C, 215.65 and 342.33$^o$C, and 198.35 and 363.64$^o$C (Figures. 21 a, b, c) in the rifampicin loaded chitosan nanoparticles. These findings indicate that physical state of rifampicin and chitosan has changed from crystallinity to amorphous state that help improved dissolution of the drug in the environment.

### 11.4 Morphology by scanning electron microscopy

Figures (22-49) shows the morphology of nanoparticles. All nanoparticle formulations prepared with different grades of chitosan at variable stirring speed and concentration of tween 80 were spherical with smooth surfaces and solid dense and showed no aggregation due to the result of stable zeta potential on the surface of the nanoparticles that prevent the agglomeration process. The morphology of all formulations was almost identical and was not affected by the molecular weight of polymer, concentration of tween 80 and stirring speed.

## 11.5 Surface characteristics by zetasizer

Table 25 shows the results data of zeta potential, zeta size, PDI of nanoparticles. Commonly, zeta potential is an index of the stability of the nanoparticles. Under most conditions, the higher the absolute value of the zeta potential of the nanoparticles, the larger the charge on their surface, leading to stronger repulsive interaction between the dispersed nanoparticles and higher stability and more uniform size[198]. A high potential value of above ±25mV, ensures a high energy barrier that stabilizes the nano suspension[199]. The zeta potential of all formulations ranged from +32mV to +42mV with a positive surface charge possibly due to the presence of amino groups in the polymer and zeta potential of all the formulations was above +25mV, the limit for the stability of nanoparticles. The zeta potential of nanoparticles was affected by molecular weight of chitosan, stirring speed, tween 80 concentration.

The zeta potential decreased significantly with increase in molecular weight of chitosan (Figure 50); conversely, the zeta potential of nanoparticles increased with increase in stirring speed and tween 80 concentrations (Figure 51). The possible mechanism may be formation of fine particles by increased stirring speed that may facilitate greater interaction between the drug and the polymer and therefore increased zeta potential of the nanoparticles. These formed nanoparticles with higher zeta potential were better stabilized with higher concentration of tween 80. Higher molecular weight chitosan may probably hinder fine particles formation by ionic gelation method and thus reduced zeta potential. Zeta potential values obtained in the present study allow predicting good colloidal stability due to high energy barrier between particles. These findings suggest chitosan molecular weight, stirring speed, tween 80 concentrations need to be optimized for the development of stable nanoparticles.

Poly dispersity index is another factor that represents the dispersion homogeneity; the range for the PDI is from 0-1.Values close to 0 indicates the homogenous dispersion and those greater than 0.5 indicate high heterogeneity. The PDI for all formulations was between 0.2-0.5 which indicates a relative homogenous dispersion[200]. Further, the homogeneity of the dispersion was influenced by the molecular weight of polymer, stirring speed and concentration of the stabilizing agent; however maintained homogeneous dispersion with PDI at or lower than 0.5.

Figure 50 shows the influence of molecular weight on particles size. Particle size plays a critical role in influencing the physicochemical and biological characteristics of nanoparticles. Smaller particles less than 400nm are most preferred in pharmaceutical product development. In the present study the particle size of all formulations ranged from 202nm-513nm, and increased significantly with increase in molecular weight of polymer. The decrease in particle size with low molecular weight chitosan is possibly due to the increased solubility of the polymer that may aid in the colloidal solubility of nanoparticles in the solution[201]. Furthermore, higher molecular weight chitosan is less soluble, and as a result an increase in particle diameter or even aggregation may be obtained.

Tween 80 as a stabilizing agent is adsorbed on to the surface of the nanoparticles, thereby slowing down the growth of crystal phases by reducing the surface free energy. It was found in the study that the nanoparticles prepared in the presence of tween 80 were much smaller, spherical particles, however the size of these particles decreased significantly as the concentration of tween 80 increased (Fig 53). Our finding is consistent with earlier report that higher concentration of tween 80 reduces the surface free energy to a great extent and hence stabilization of the smaller particles formed[202]. Besides, stirring speed also has produced the same effect of tween80 on the particle size of nanoparticles in our study. As the stirring speed increased the particle size of the nanoparticles decreased significantly (Figure 54). This effect is due to the occurrence of external energy and thus the shear stress causing droplet breakdown increased with increased stirring speed [203].

## 11.6 Rifampicin encapsulation efficiency and loading capacity of nanoparticles

Results data of rifampicin encapsulation efficiency and loading capacity of the nanoparticles are given in Table 26. The drug encapsulation efficiency and loading capacity of the nanoparticles depend upon the molecular weight of the polymer, the interaction between the drug and the polymer, the stirring speed used and also the concentration of the stabilizing agent added in the development of nanoparticles. Figures 55, 56 shows that the increase in the molecular weight of chitosan, the stirring speed and the concentration of tween 80 (stabilizing agent), all resulted increase in the encapsulation efficiency and loading capacity significantly. Rifampicin having carboxyl group resulted in electrostatic interaction with the amino group of chitosan and therefore influenced the encapsulation efficiency and loading capacity of nanoparticles. High molecular weight chitosan has more reaction sites and therefore more electrostatic interaction with rifampicin. Our findings consistent with earlier observation that longer chains of high molecular weight chitosan can entrap greater amount of drug when gelated with tripolyphosphate[204]. Further increasing stirring speed or concentration of tween 80 brings about breaking down of the particles to smaller size caused by increased shearing stress and consequently stabilization of the formed nanoparticles by higher concentration of the stabilizing agent which promotes greater interaction between the drug and polymer and therefore increased encapsulation efficiency and loading capacity of the nanoparticles (Figure. 57).

### 11.7 *In-vitro* dissolution stability

The results of *in-vitro* percentage degradation of rifampicin with isoniazid / ascorbic acid and of nanoparticles are shown Table 43. Rifampicin degraded 33.1% at 15 min, and the degradation increased overtime and reached 42.7% at 60min and our finding supports the view that rifampicin undergoes degradation in the acidic environment of the stomach and the degradation has varied from 8.5%-50% during the gastric emptying time for most dosage form in humans[4]. Rifampicin degrades to 3-formyl rifampicin SV (3FRSV) and 1-amino 4 methyl piperazine under acidic pH[69] and at the same time it is more soluble at low pH and therefore well absorbed from the stomach because of its high solubility between pH1 and 2[205]. The degradation of rifampicin increased to 74% in the presence of isoniazid in the simulated gastric fluid and this increase was statistically significant ($P<0.01$) and in consistent with earlier claim that decomposition of rifampicin is further influenced by the presence of isoniazid after ingestion[16]. Ascorbic acid as an antioxidant protects rifampicin from degradation to 3FRSV in the plasma sample[3]. In the present study ascorbic acid in different concentrations (125mg, 250mg, 500mg, and 1000mg) was used to examine its protective effect against degradation in the simulated gastric fluid. Ascorbic acid addition reduced the rifampicin degradation at all time point intervals and the percent drug degradation decreased significantly as the concentration of ascorbic acid increased and drug degradation reached minimum (20.40%) with 500mg ascorbic acid and there was no further significant decrease in drug degradation beyond this concentration of ascorbic acid. At 60min, the degradation of drug decreased from 42.7% to 20.40% significantly ($P<0.01$) in the presence of ascorbic acid (500mg). Besides ascorbic acid also minimized rifampicin degradation in the presence of isoniazid from 74% to 25.6% and this effect was statistically significant ($P<0.001$).

Nanoparticles being small in size can help enhanced dissolution and improve the bio-availability of the drugs. Applying this factor the present study attempted to examine whether nanoparticle system can help reduce the degradation of rifampicin in the simulated gastric fluid (without enzyme). Rifampicin nanoparticles degraded significantly less than rifampicin at all time point intervals and at 60min it degraded 32.15% as compared to rifampicin (42.7%) ($P<0.05$). Nanoparticle delivery of rifampicin reduced rifampicin degradation from 74% to 56.76% in the presence isoniazid at 60min. Ascorbic acid significantly reduced degradation of rifampicin nanoparticles at all time intervals and, at 60min from 32.15% to 25.92%. Rifampicin – ascorbic acid nanoparticles showed enhanced degradation of drug (25.92%) as compared to rifampicin - ascorbic acid (20.4%), possibly, nanoparticles give rise to larger surface area becoming available for interaction with acidic environment that may facilitate degradation of rifampicin. However nanoparticulate delivery of rifampicin along with ascorbic acid has reduced degradation from 42.7% to 25.92% and ascorbic acid addition to rifampicin nanoparticle in the presence of isoniazid reduced rifampicin degradation significantly from 25.6% to 20.87% and this finding supports the view that nanopaticle approach seems beneficial in overcoming degradation of rifampicin in the acidic environment of the stomach. Additionally ascorbic acid could further protect rifampicin from degradation in the acidic environment. Though the literatures report that ascorbic acid can stabilize rifampicin against degradation either in the plasma sample or in the dissolution medium, the underlying mechanism of protection by ascorbic acid against rifampicin degradation in the presence of isoniazid in the acidic environment is not clearly established. In the present study too the results obtained with ascorbic acid co-administration could not explore the underlying mechanism of stabilization of rifampicin by ascorbic acid. However it has been documented that rifampicin, a zwiterionic compound at low pH the basic piperezine nitrogen group of rifampicin become protenated with positive charge leading to cationic nitrogen having strong tendency to form ion pair with sodium lauryl sulphate[72]. It is hypothesized whether such a mechanism is feasible where in the rifampicin possibly forms a soluble complex with ascorbic acid and thus protects rifampicin against degradation in the acidic environment.

To support the above said mechanism the following points need to be addressed: Rifampicin is freely soluble at pH 1-2 and the solubility decreases with increase in pH. In clinical practice rifampicin is recommended to be taken on empty stomach in order to ensure the bioavailability of rifampicin. On addition of ascorbic acid, the shift in pH less than 1-2 is more likely by the effect of ascorbic acid. The mechanism underlying improved stability of rifampicin in the acidic environment either by ascorbic acid or by nanoparticles and ascorbic acid together cannot be clearly ascertained.

### 11.8 *In-vitro* diffusion study

The results of *in-vitro* diffusion study are shown in Table 52. The release of rifampicin was 32.4% at 15 min and increased over time and reached 48.6% at 60 min. Ascorbic acid significantly improved the release of rifampicin at all time point intervals, and at 60 min, from 48.6% to 67.21%. This finding is consistent with our observation in the dissolution stability study and suggests that ascorbic acid may overcome the degradation and enhance the absorption of rifampicin by further lowering the acidic pH at which the drug is more soluble. The percentage release of rifampicin was 48.6% which was reduced significantly to 18.1% in the presence of isoniazid due to promotion of degradation by isoniazid. Ascorbic acid significantly increased percentage release of rifampicin in the presence of isoniazid from 18.1% to 60% indicating the stabilizing effect of ascorbic acid against rifampicin degradation in the acidic environment, besides protecting rifampicin from isoniazid induced degradation of rifampicin. Rifampicin nanoparticles increased release of rifampicin significantly over time as compared to rifampicin and released 67.23% at 60 min as compared to rifampicin (48.6%), possibly, rifampicin when presented in nanosize may improve the transmucosal transport of the drug through the membrane at a rate faster than rate of degradation of drug in the acidic environment, besides protecting the drug from the degradation in the acidic environment. This was further evident that nanoparticles of rifampicin increased the drug release from 18.1% to 38.83%, thus showing the stabilization effect of nanoparticles against rifampicin degradation under the influence of isoniazid. The stabilizing effect of ascorbic acid against rifampicin degradation was further improved significantly when rifampicin was presented as nanoparticles, and the percentage release of rifampicin increased significantly from 67.21% to 74.01% ($P<0.05$). Similar effect was observed with rifampicin-ascorbic acid nanoparticles containing isoniazid as compared to rifampicin-ascorbic acid containing isoniazid. These findings propose the hypothesis that nanoparticles coupled with ascorbic acid may promote absorption of rifampicin faster than its degradation of rifampicin alone as well as in the presence of isoniazid. Besides, chitosan being mucoadhesive may also play a role in promoting absorption of rifampicin.

### 11.9 *In-vivo* pharmacokinetic study

*In-vivo* pharmacokinetic parameters were studied on the best formulation (F8) selected, based on the particle size, the percentage degradation, percentage release and encapsulation efficiency of rifampicin nanoparticles and the results are shown in Table 69. The study was carried out in rabbits in order to ascertain whether the *in-vitro* release and degradation data of rifampicin and rifampicin nanoparticles would be truly reflected in *in-vivo* bioavailability. HPLC chromotograms and graphical representation of the data are shown in Figures 60-75.

Isoniazid reduced $C_{max}$ from 5.9 µg/ml to 4.1µg/ml, $AUC_{0-12}$ (µg/mlh) from 33.96 to 27.80 and $AUC_{0-\infty}$(µg/mlh) from 61.05 to 53.53 and these reductions are statistically significant ($P<0.05$) and these changes are due to degradation of rifampicin under the influence of isoniazid and therefore reduction in bioavailability of rifampicin. As compared to rifampicin, ascorbic acid addition improved $C_{max,}$ $AUC_{0-12}$ (µg/mlh) and $AUC_{0-\infty}$ (µg/mlh) significantly from 5.9µg/ml to 9.3µg/ml ($P<0.01$), 33.96 to 66.14 ($P<0.01$) and 61.05 to 82.48 ($P<0.01$) respectively. These findings reveal the stabilizing effect of ascorbic acid against rifampicin degradation in the stomach as observed in the *in-vitro* dissolution stability and diffusion studies. Ascorbic acid also stabilized rifampicin against degradation in the presence of isoniazid and the $C_{max}$ of rifampicin increased from 4.1µg/ml to 10.2µg/ml, $AUC_{0-12}$ (µg/mlh) from 27.81 to 71.42 and $AUC_{0-\infty}$ (µg/mlh) from 53.53 to 89.54. These findings reveal the influence of ascorbic acid protecting isoniazid induced rifampicin degradation in the stomach and increasing the concentration of drug available for absorption. $K_{el}$ of rifampicin was decreased by ascorbic acid and the cause underlying this could not be ascertained; whether ascorbic acid increases residence time of the drug in the blood and so $K_{el}$ is reduced, needs to be investigated. As $AUC_{0-12}$ and $AUC_{0-\infty}$ were increased $V_d$ was found decreased. $T_{max}$ was increased due to increase in $C_{max}$. The effect of nanoparticle approach and ascorbic acid addition together on the bio-availability of rifampicin was also examined. These findings clearly propose that ascorbic acid can improve the bio-availability of rifampicin irrespective of the presence and absence of isoniazid at the absorption site.

The effect of nanoparticle approach and how it was influenced by ascorbic acid on the bio-availability of rifampicin was also examined. Rifampicin delivery as nanoparticle improved $C_{max}$ $AUC_{0-12}$(µg/mlh) and $AUC_{0-\infty}$ (µg/mlh) significantly as compared to rifampicin. The $C_{max}$, AUC $_{0-12}$(µg/mlh) and $AUC_{0-\infty}$ (µg/mlh) of rifampicin increased significantly from 5.9µg/ml to 6.3µg/ml ($P<0.05$), 33.96 to 63.69 ($P<0.01$) and 61.05 to 68.79 ($P<0.05$) significantly from the nanoparticles.

However isoniazid reduced the stabilizing effect of nanoparticle approach as the $C_{max}$, $AUC_{0-12}$ (µg/mlh) and $AUC_{0-\infty}$ (µg/mlh) were significantly ($P<0.01$) reduced from 6.3µg/ml to 5.5 µg/ml, 63.69 to 53.76 and from 68.70 to 58.34 respectively. Ascorbic acid improved the stability of rifampicin nanoparticles significantly as evident from the $C_{max}$, $AUC_{0-12}$(µg/mlh) and $AUC_{0-\infty}$ (µg/mlh). The $C_{max}$ of rifampicin increased from 6.3µg/ml to 10.1µg/ml ($P<0.01$), $AUC_{0-12}$ from 63.69 to 70.35 ($P<0.01$) and $AUC_{0-\infty}$ from 68.79 to 79.92 ($P<0.01$). Similarly ascorbic acid protected rifampicin from degradation under the influence of isoniazid enhancing the $C_{max}$, $AUC_{0-12}$ (µg/mlh) and $AUC_{0-\infty}$ (µg/mlh) values. The $C_{max}$, $AUC_{0-12}$ (µg/mlh) and $AUC_{0-\infty}$ (µg/mlh) of rifampicin with isoniazid were 5.5µg/ml, 55.76, and 58.74 respectively and these values were increased to 10.4, 71.78, and

80.61µg/ml respectively. There were corresponding changes in the $K_{el,}$ Ka, $V_d$, $t_{1/2}$ and $T_{max}$ and these changes were statistically significant ($P<0.05$). The results of the *in-vivo* study clearly suggest that ascorbic acid can stabilize rifampicin against degradation in the acidic environment following oral ingestion. Furthermore ascorbic acid coupled with nanoparticle delivery of rifampicin can greatly overcome the rifampicin degradation and improve its bio-availability significantly.

The beneficial effect of ascorbic acid in tuberculosis has also been well documented. Ascorbic acid, as antioxidant and immune enhancing nutrient when used in conjuction with standard TB regimen accelerate healing from tuberculosis[206]. An ascorbate concentration of 1mg/day, which is easily reached in the blood, prevents the growth of cultures of *M.tuberculosis*[207]. TB patients are found to have elevated MMP-9 levels[208], which is correlated with severity of illness in patients with active tuberculosis[209]. MMP-9 is secreted by both the bacillus and host response to infection with ascorbic acid is critical for inhibiting the activity of plasmin and matrix mettalloproteinasses (mmp-2 and mmp-9) and for maintaining optimum synthesis and structure of the connective tissue which undergoes enzymatic destruction in TB infection. It has been demonstrated that adequate levels of ascorbic acid can prevent MMP-9 secretion and degradation of collagen matrix in various system[208].

# 12. SUMMARY AND CONCLUSION

The present study followed two directional approaches to improve the disadvantage of rifampicin degradation that affects bio-availability of the drug.

- In the first approach ascorbic acid was used to stabilize rifampicin against degradation in the acidic environment and the second approach is to present rifampicin nanoparticles along with ascorbic acid.
- Ascorbic acid significantly reduced rifampicin degradation both in the presence and absence of isoniazid. Ascorbic acid and the nanoparticle approach together further influenced stability of rifampicin against degradation in the acidic environment.

  The stability of rifampicin from the above approaches was reflected in reduced degradation and increased release of rifampicin in the acidic environment. *In-vivo* study revealed improved pharmacokinetics of rifampicin from nanoparticles coupled with ascorbic acid.

In conclusion, the results of the present study demonstrate that degradation of rifampicin in combination with isoniazid in fixed dose formulations can be controlled by using ascorbic acid as stabilizing agent in appropriate concentration. It has been established that tuberculous patients are recommended supplementation of ascorbic acid (1000mg/day) to improve the immune system against tuberculosis and therefore our study justifies the recommendation of ascorbic acid addition to rifampicin formulations in order to control degradation and improve bioavailability of rifampicin following oral ingestion for effective management of tuberculosis. Extrapolation of the findings of the present study to humans, may address the clinical implications of this approach.

# 13. RECOMMENDATION

The results of the study clearly demonstrated the beneficial effects of ascorbic acid co-administration in improving bioavailability of rifampicin that can help control TB effectively. Further study is recommended for preclinical and clinical outcome and thus its viability in the design of fixed dose combination of rifampicin, isoniazid, pyrazinamide, ethambutol including ascorbic acid for better management of TB infection.

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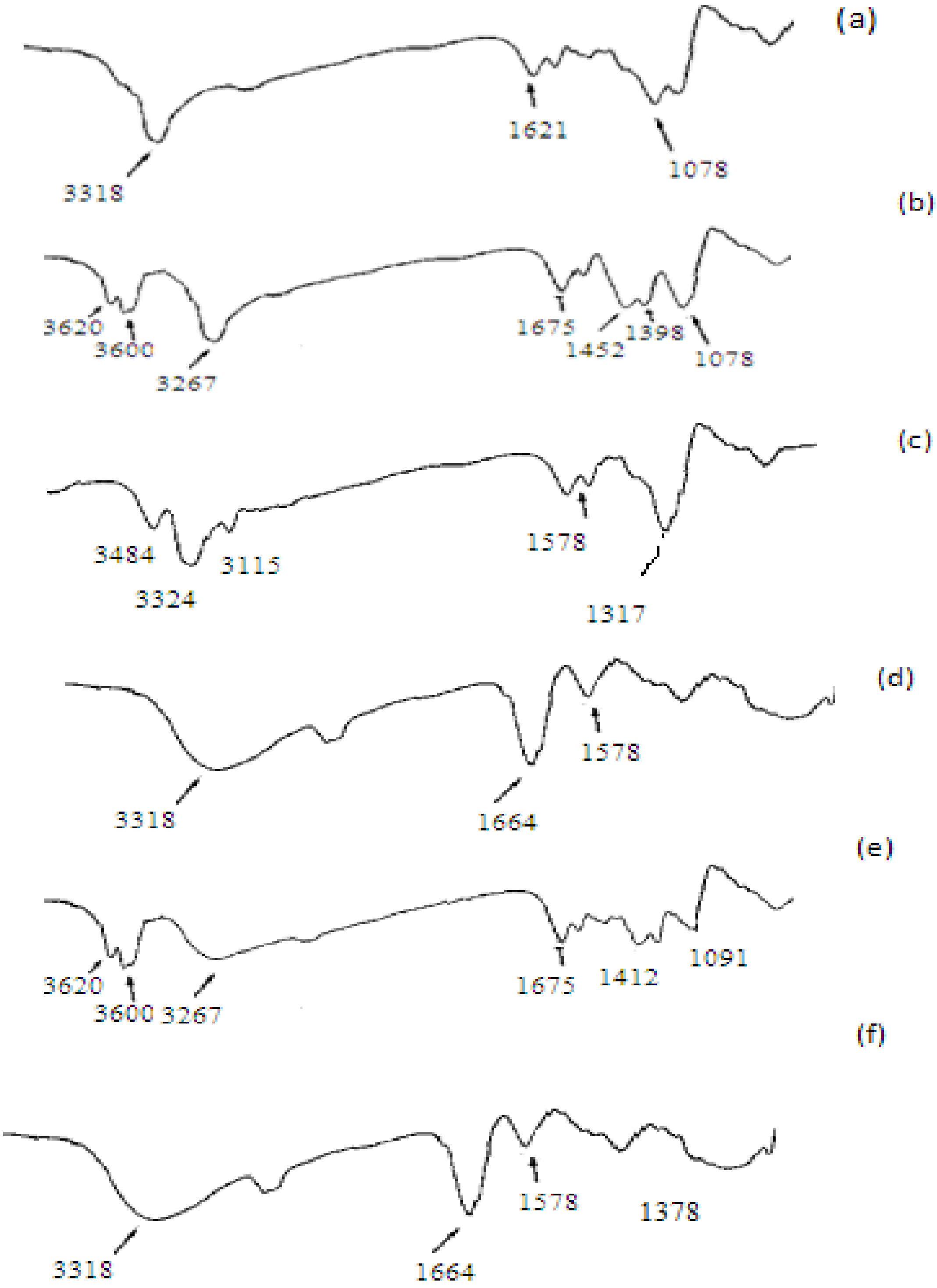


**Figure 18. FTIR of Low (a), medium(b),high(c) molecular weight chitosan, chitosan low(d), medium(e), high(f) molecular weight -TPP nanoparticles.**

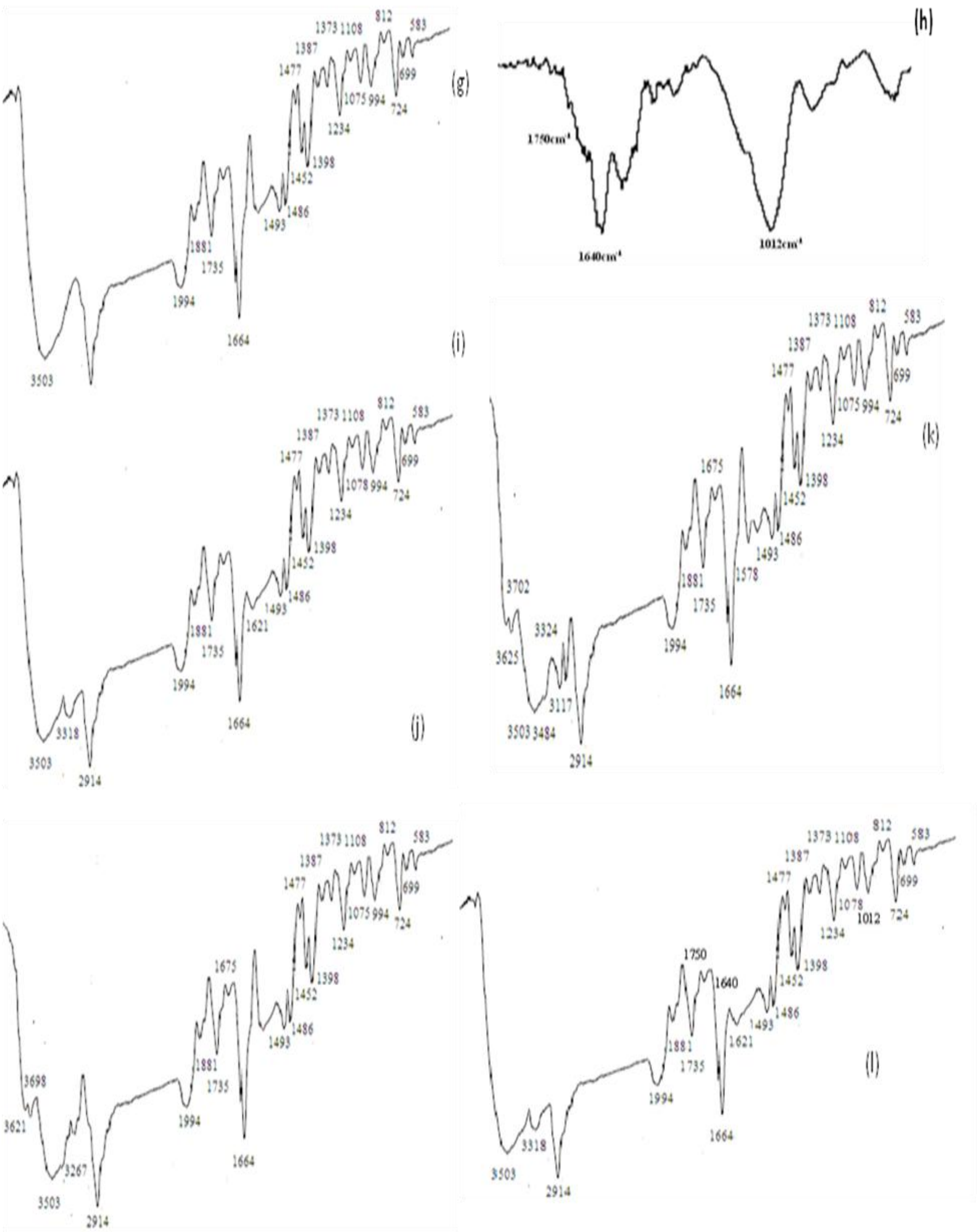


**Figure 19. FTIR of Rifampicin (g), ascorbic acid (h) and rifampicin loaded low(i), medium(j), high(k) molecular weight chitosan nanoparticle, rifampicin-ascorbic acid loaded low molecular weight chitosan nanoparticles (l)**

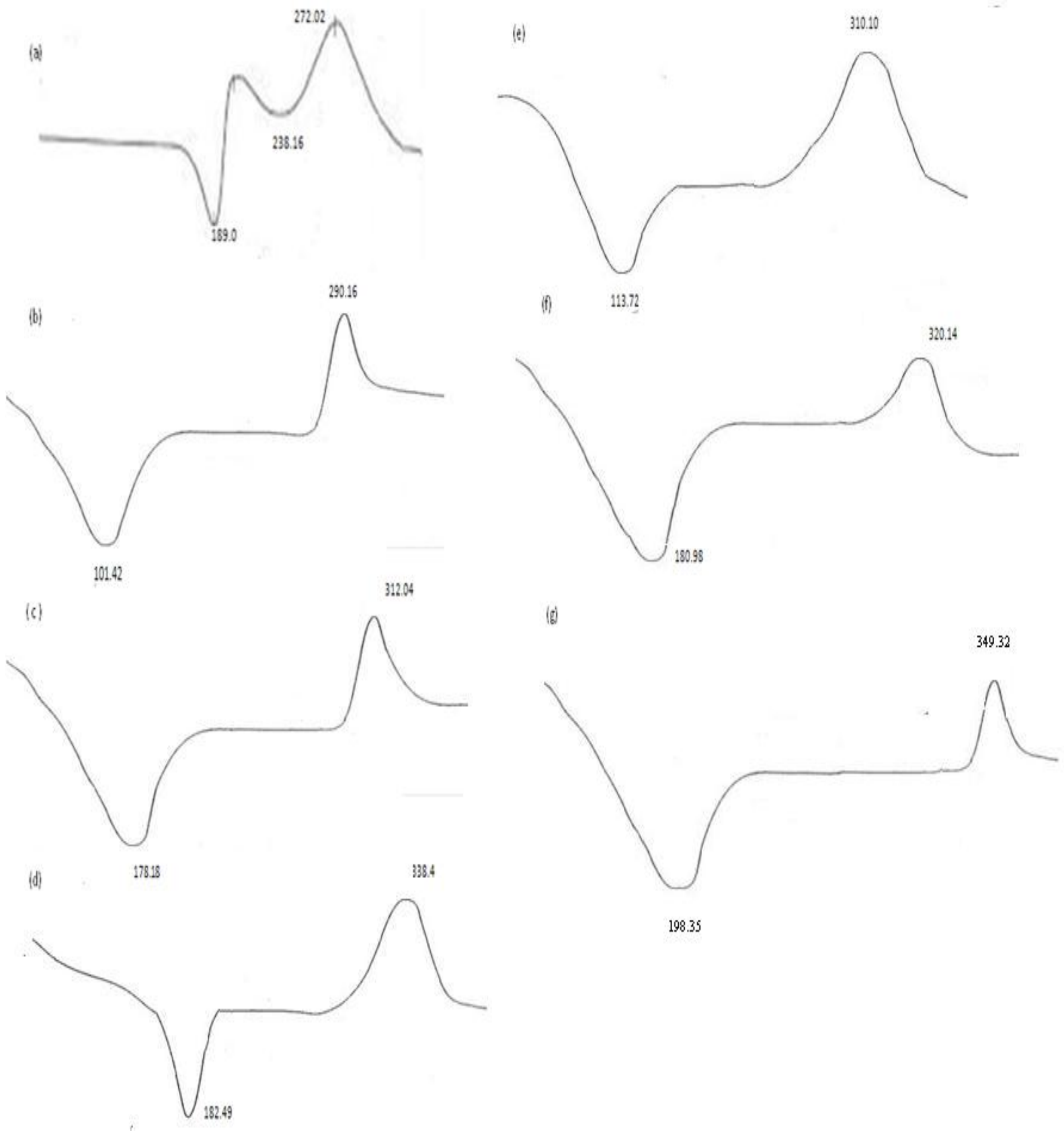


**Figure 20. DSC of Rifampicin (a), Low (b), medium(c), high (d) molecular Weight chitosan, low (e), medium (f), high(g) molecular weight chitosan nanoparticles**

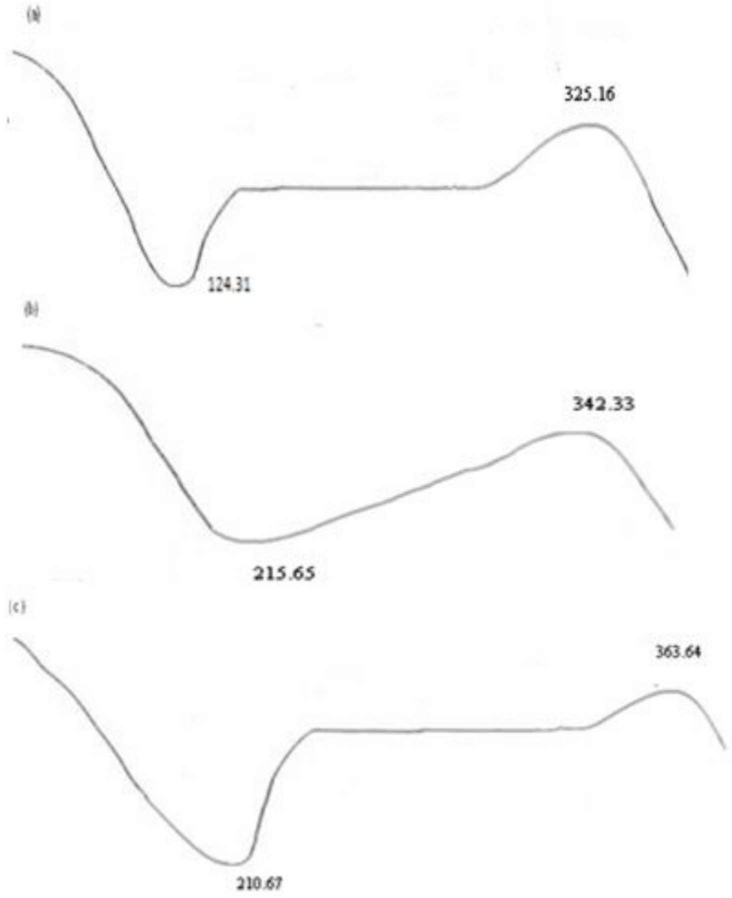


**Figure 21. DSC of low (a), medium (b), high (c) molecular weight chitosan-rifampicin nanoparticles**

**Morphology by scanning electron microscopy of rifampicin loaded chitosan nanoparticles**

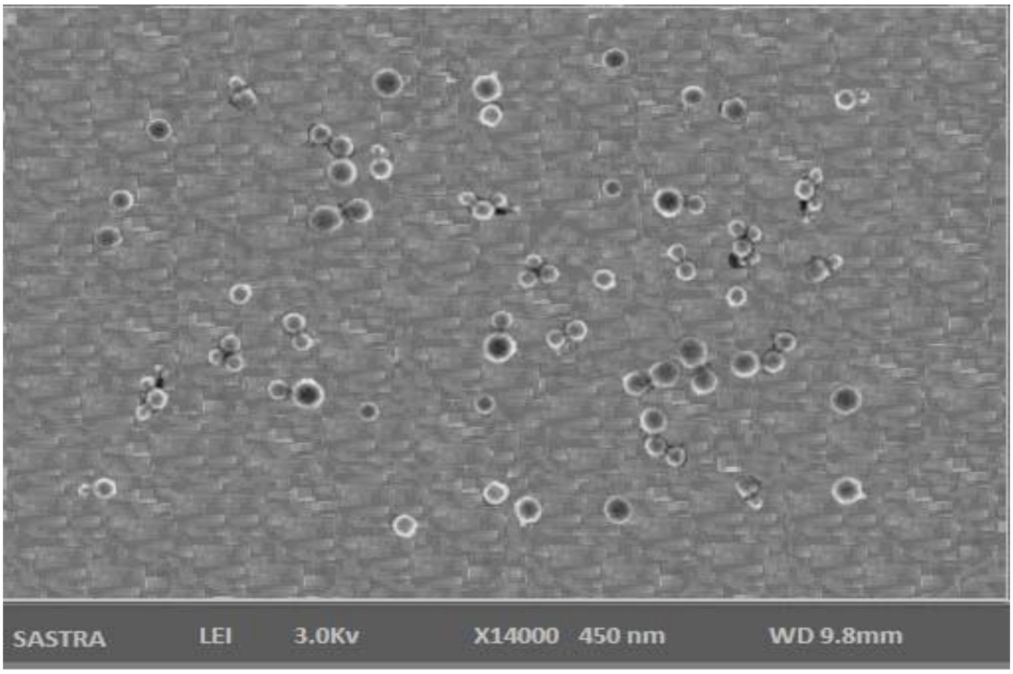


**Figure 22. Scanning electron microscopic (SEM) image of nanoparticles (F1)**

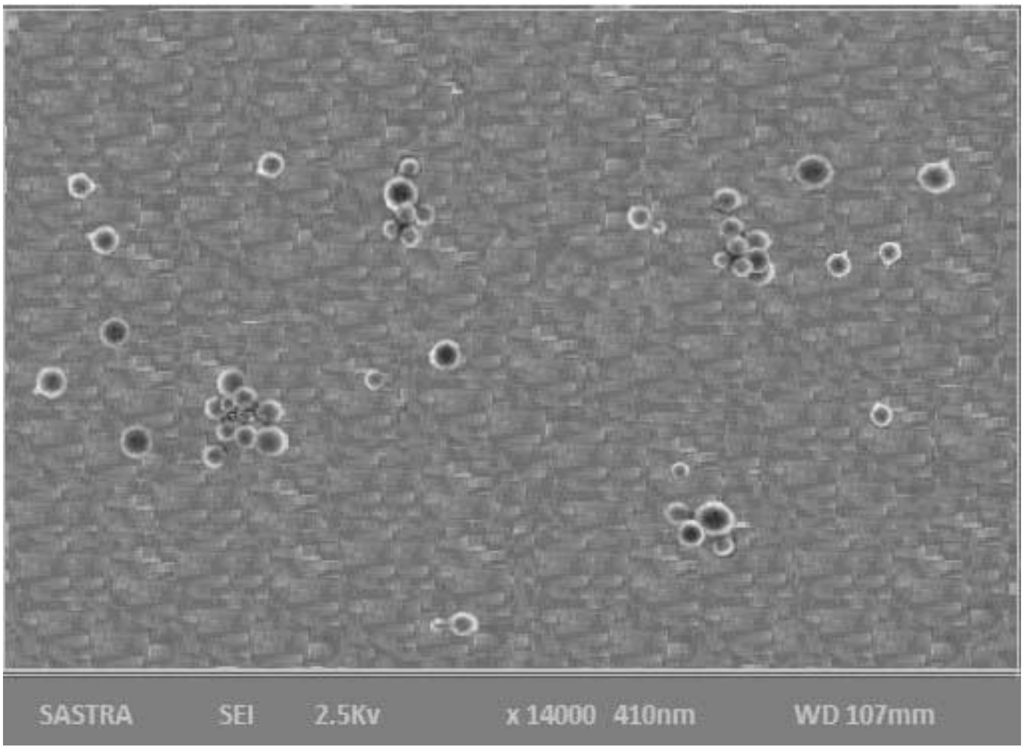


**Figure 23. Scanning electron microscopic (SEM) image of nanoparticles (F2)**

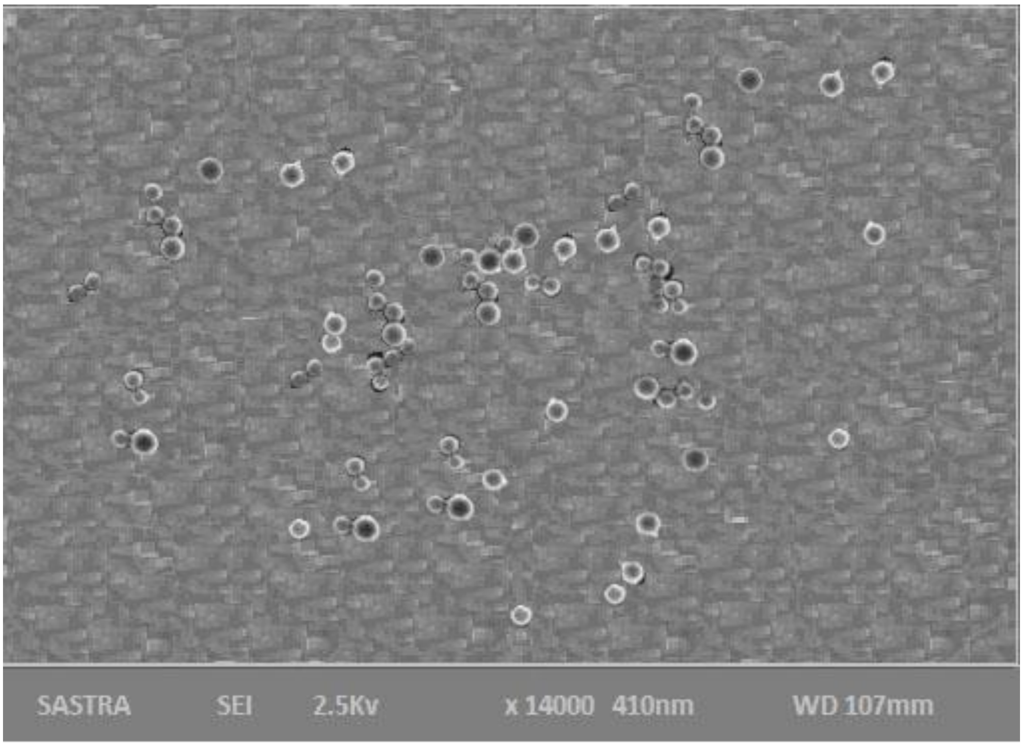


**Figure 24. Scanning electron microscopic (SEM) image of nanoparticles (F3)**

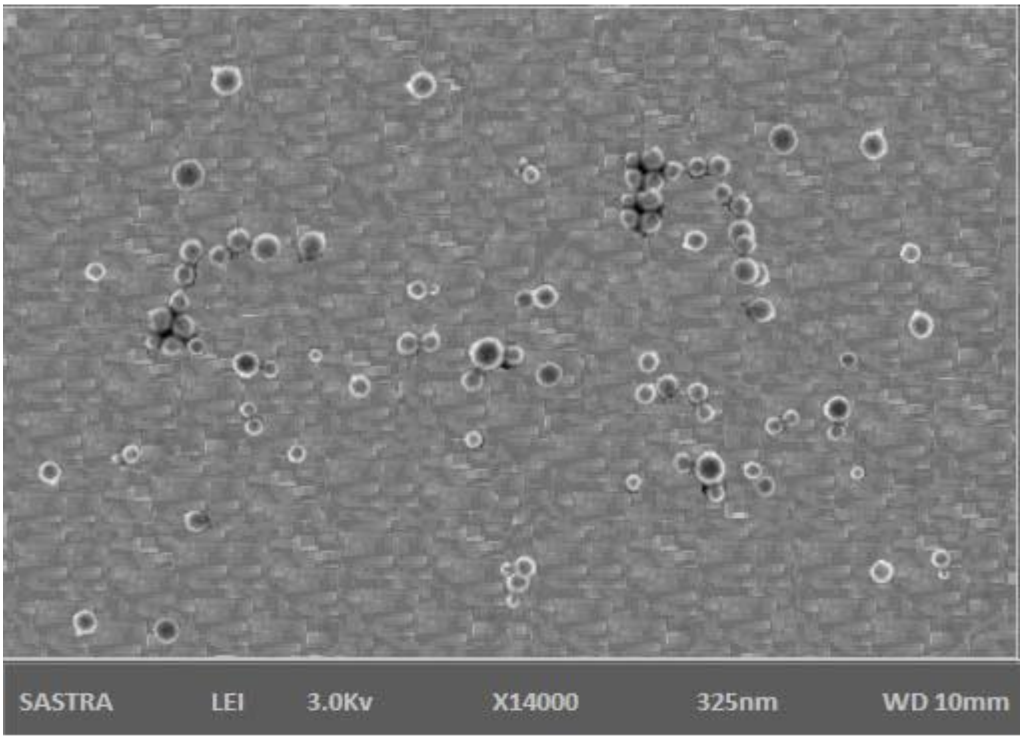


**Figure 25. Scanning electron microscopic (SEM) image of nanoparticles (F4)**

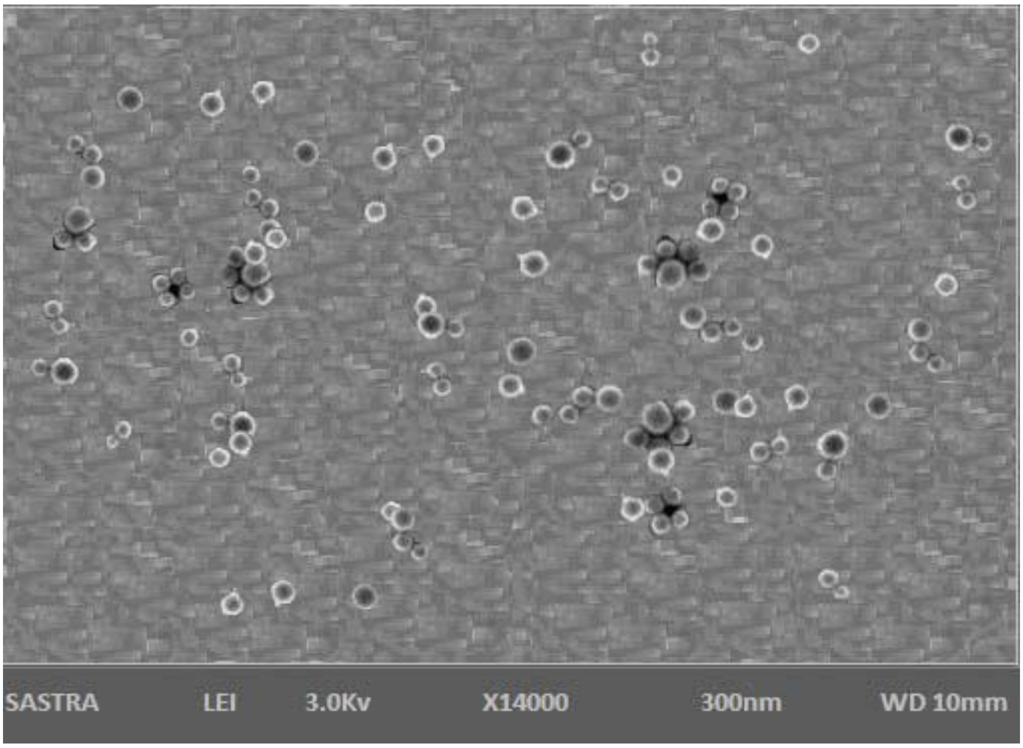


**Figure 26. Scanning electron microscopic (SEM) image of nanoparticles (F5)**

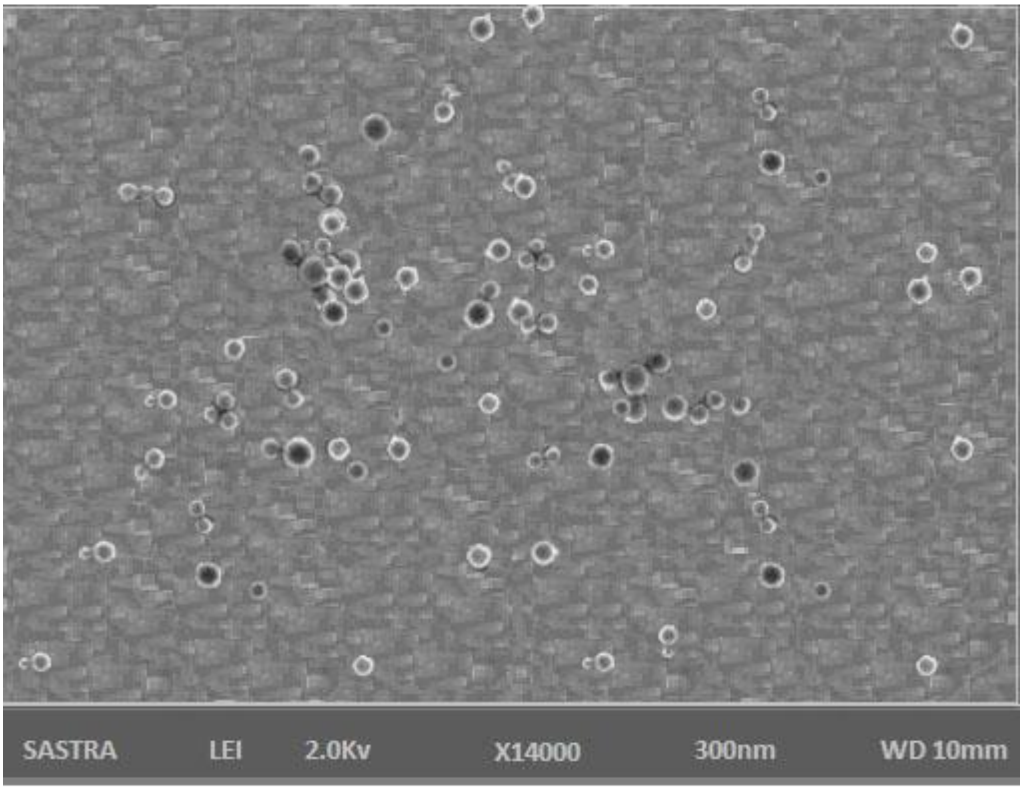


**Figure 27. Scanning electron microscopic (SEM) image of nanoparticles (F6)**

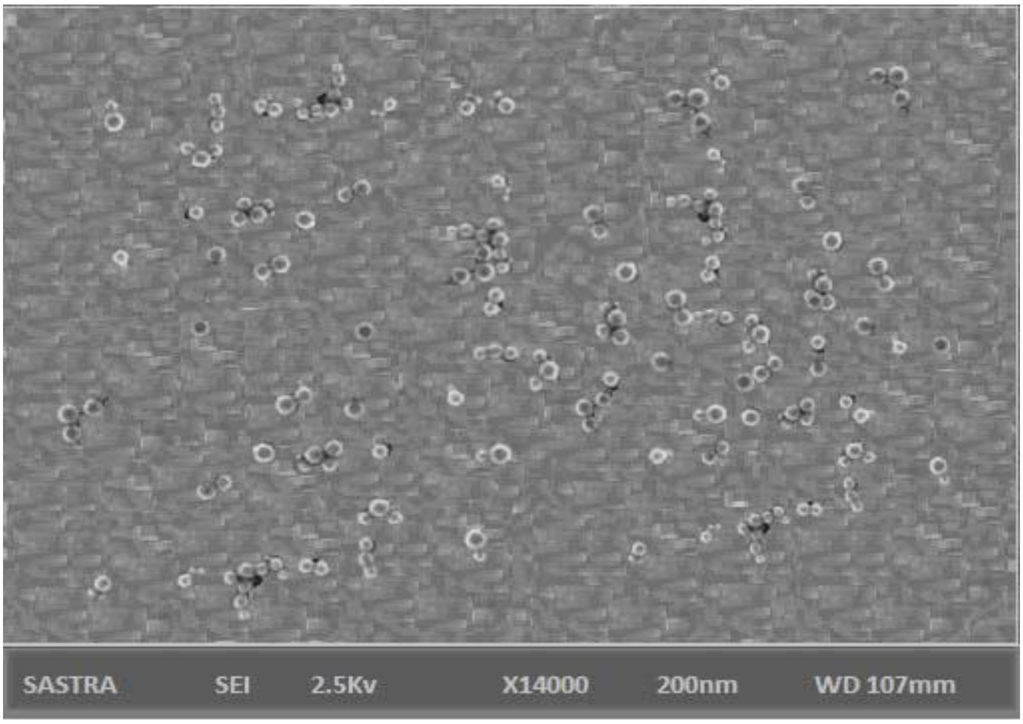


**Figure 28. Scanning electron microscopic (SEM) images of nanoparticles (F7)**

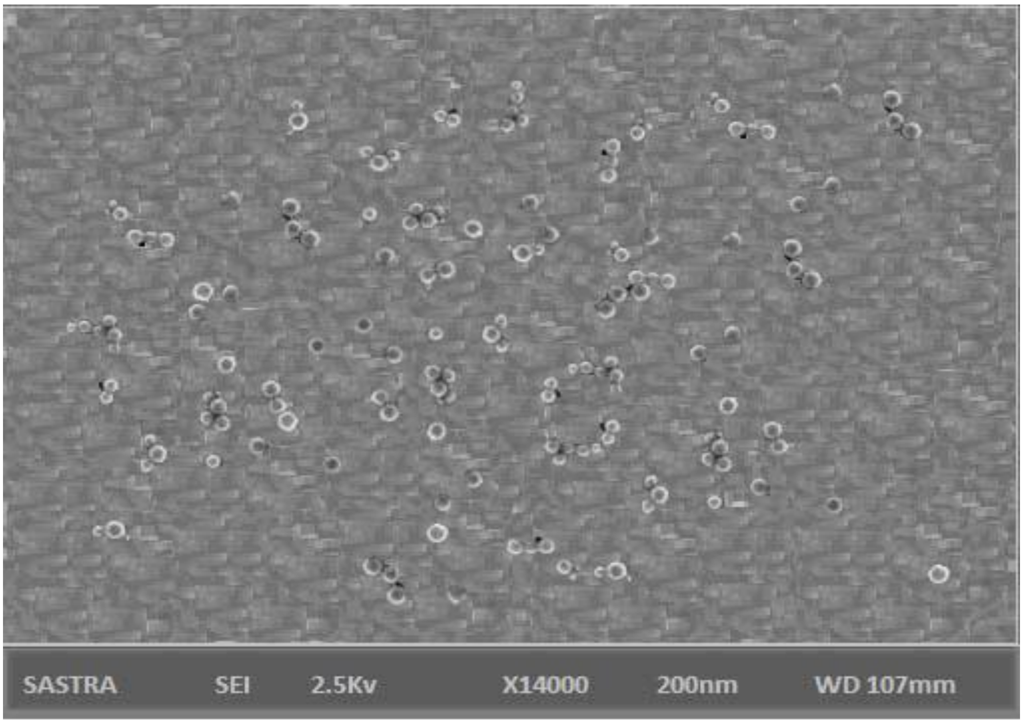


**Figure 29. Scanning electron microscopic (SEM) image of nanoparticles (F8)**

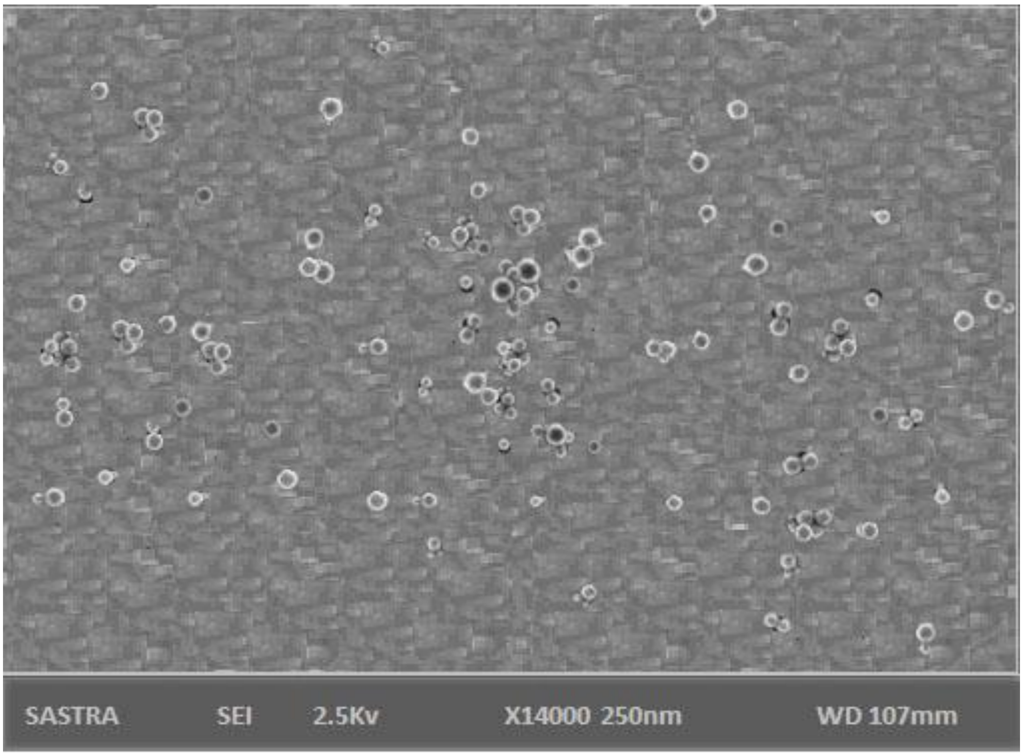


**Figure 30. Scanning electron microscopic (SEM) image of nanoparticles (F9)**

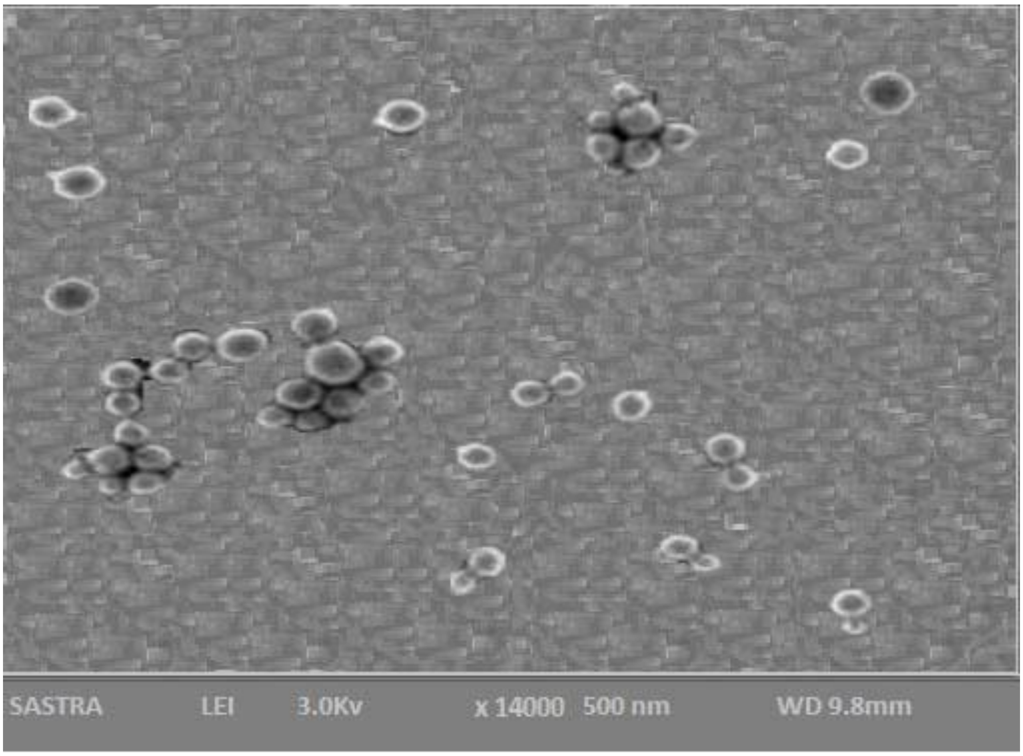


**Figure 31. Scanning electron microscopic (SEM) image of nanoparticles (F10)**

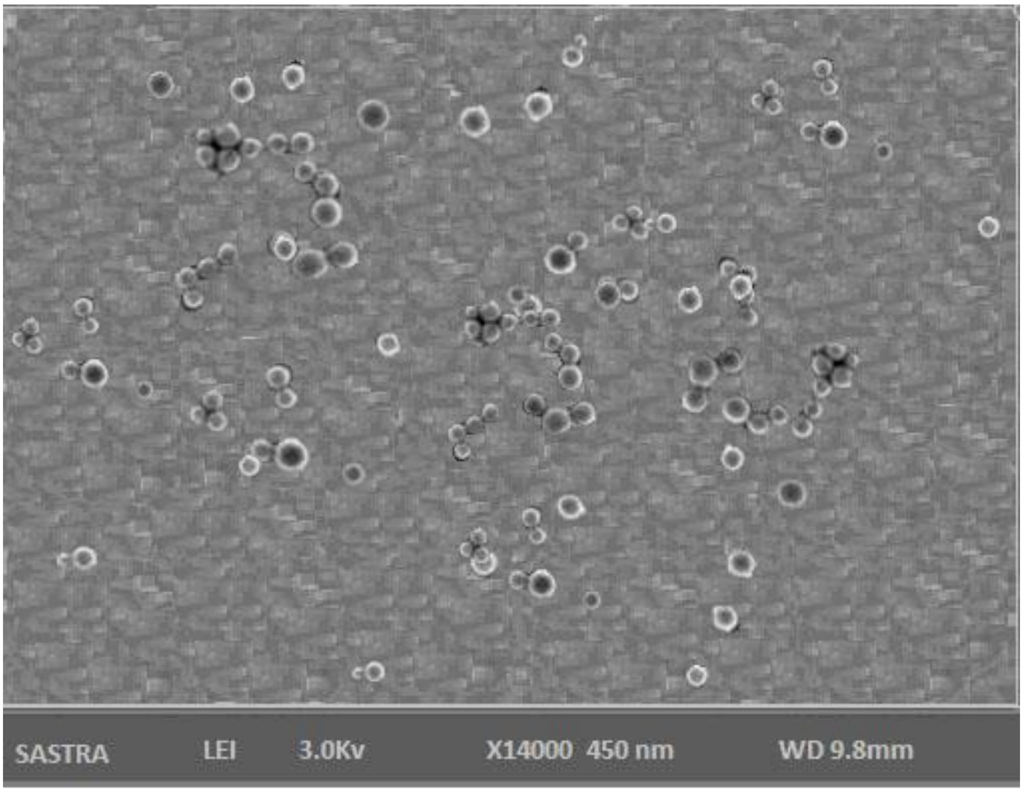


**Figure 32. Scanning electron microscopic (SEM) image of nanoparticles (F11)**

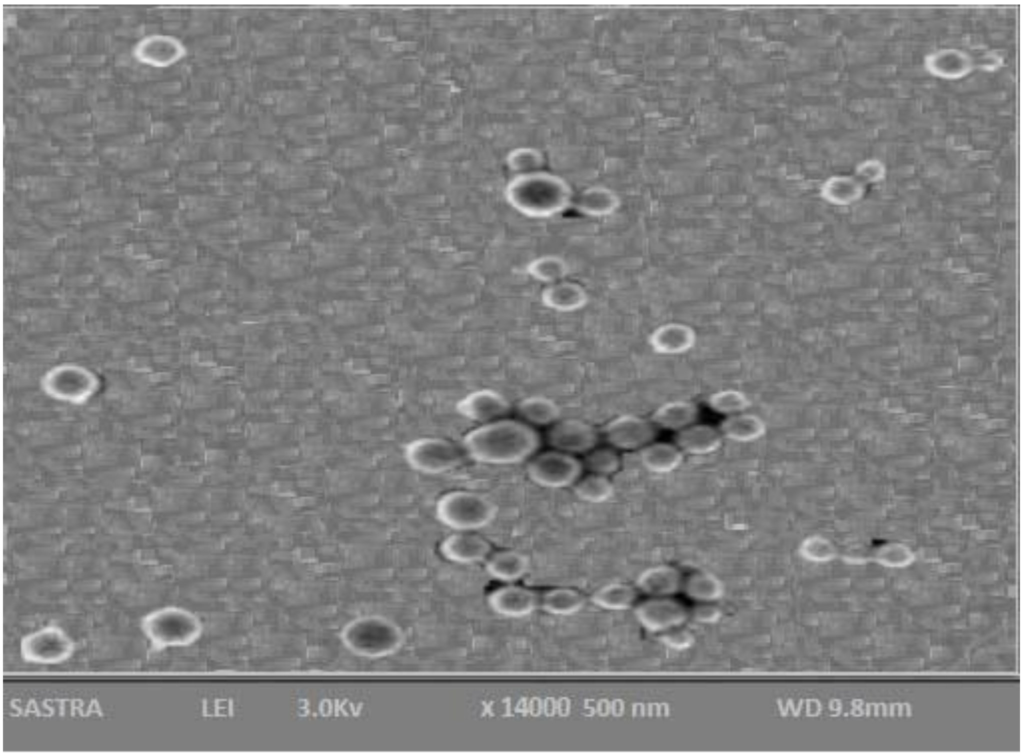


**Figure 33. Scanning electron microscopic (SEM) image of nanoparticles (F12)**

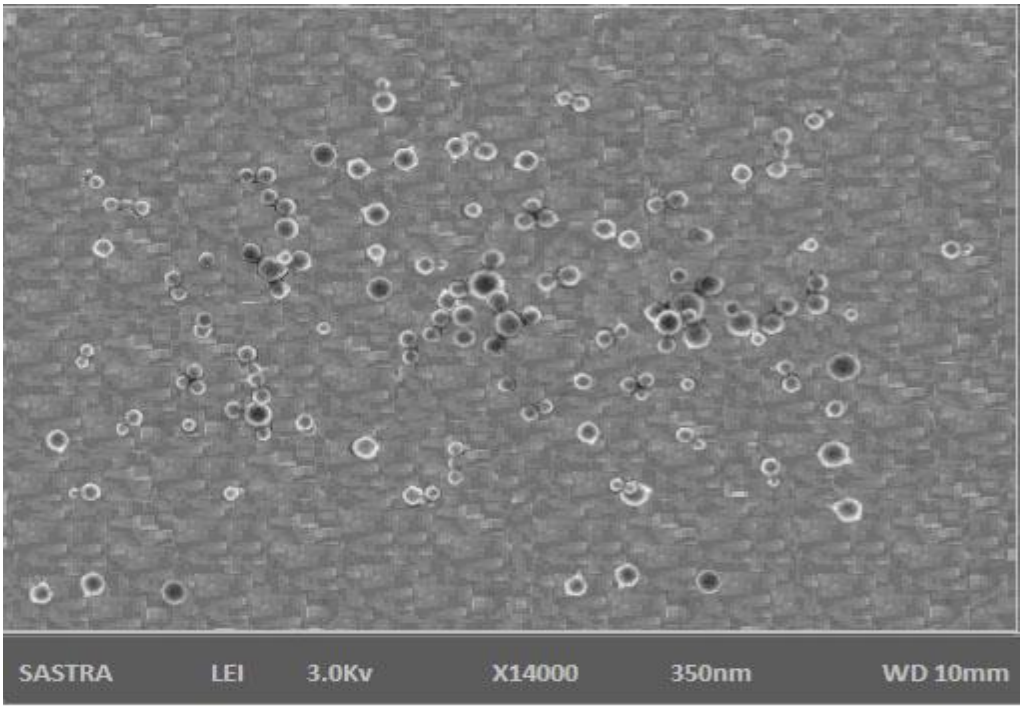


**Figure 34. Scanning electron microscopic (SEM) image of nanoparticles (F13)**

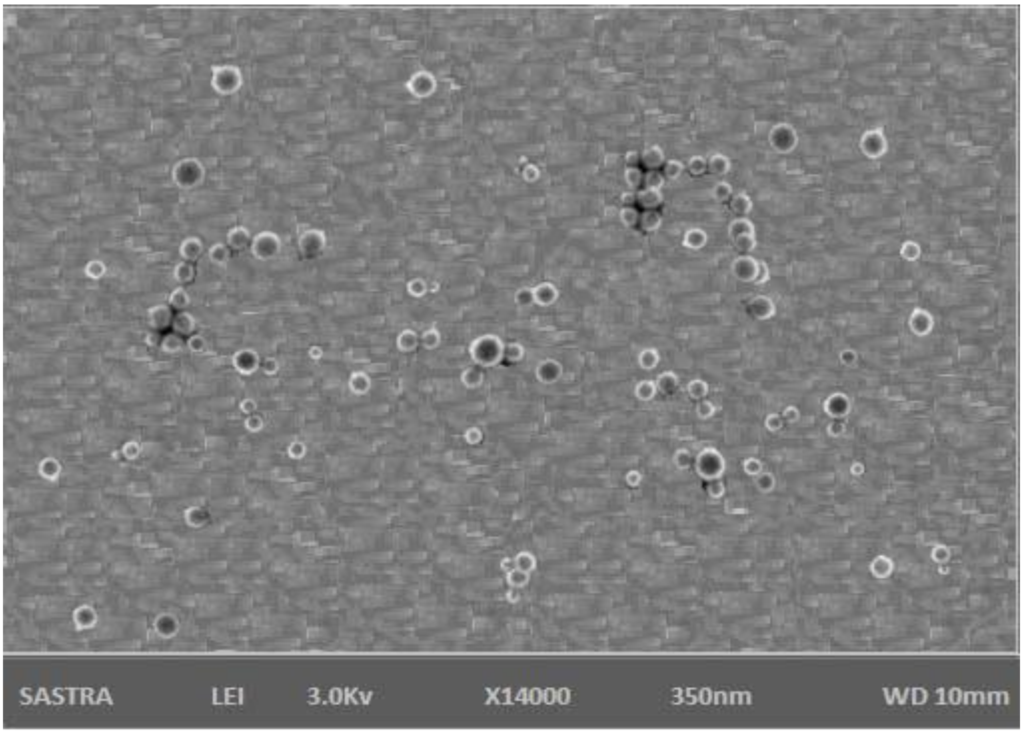


**Figure 35. Scanning electron microscopic (SEM) image of nanoparticles (F14)**

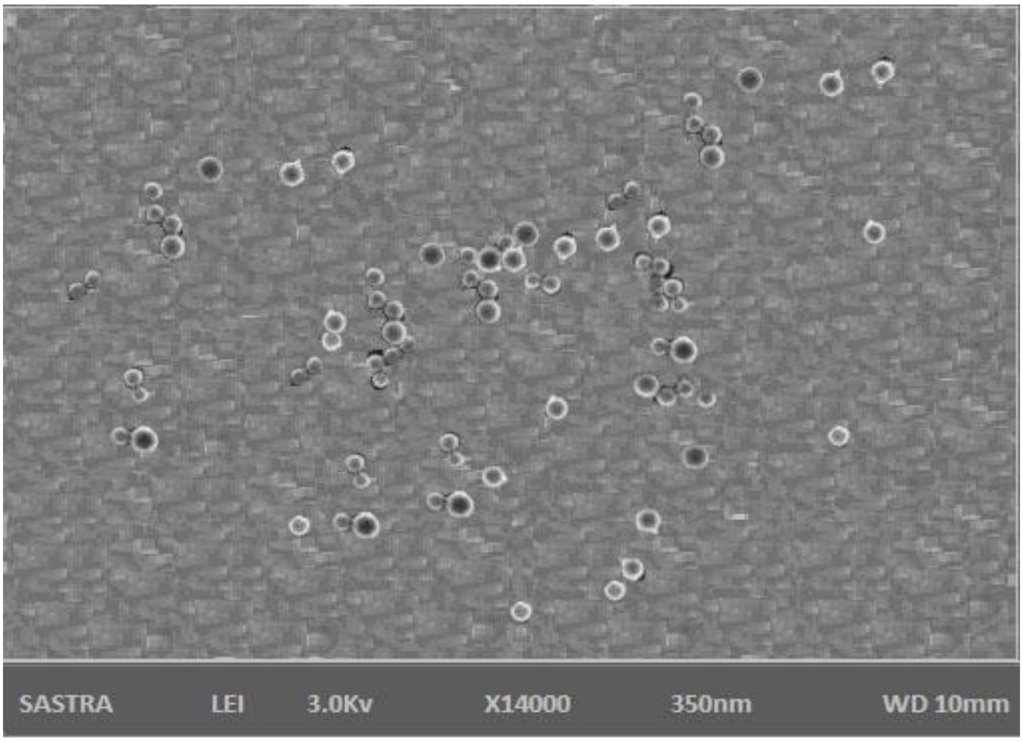


**Figure 36. Scanning electron microscopic (SEM) image of nanoparticles (F15)**

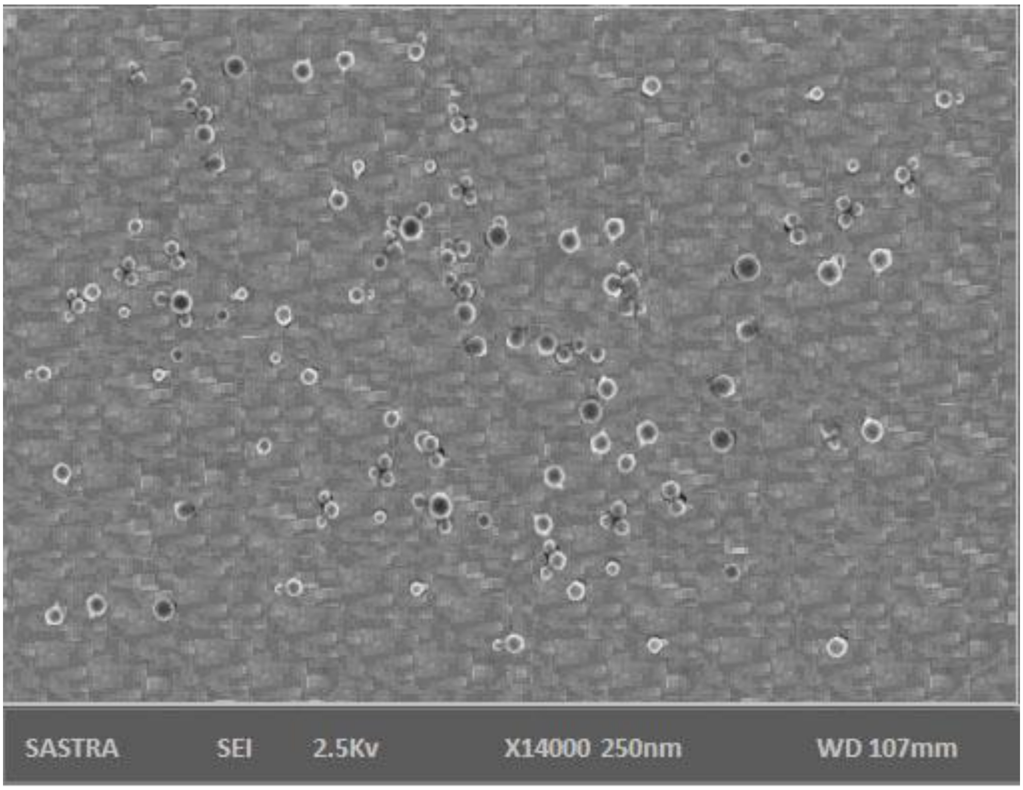


**Figure 37. Scanning electron microscopic (SEM) image of nanoparticles (F16)**

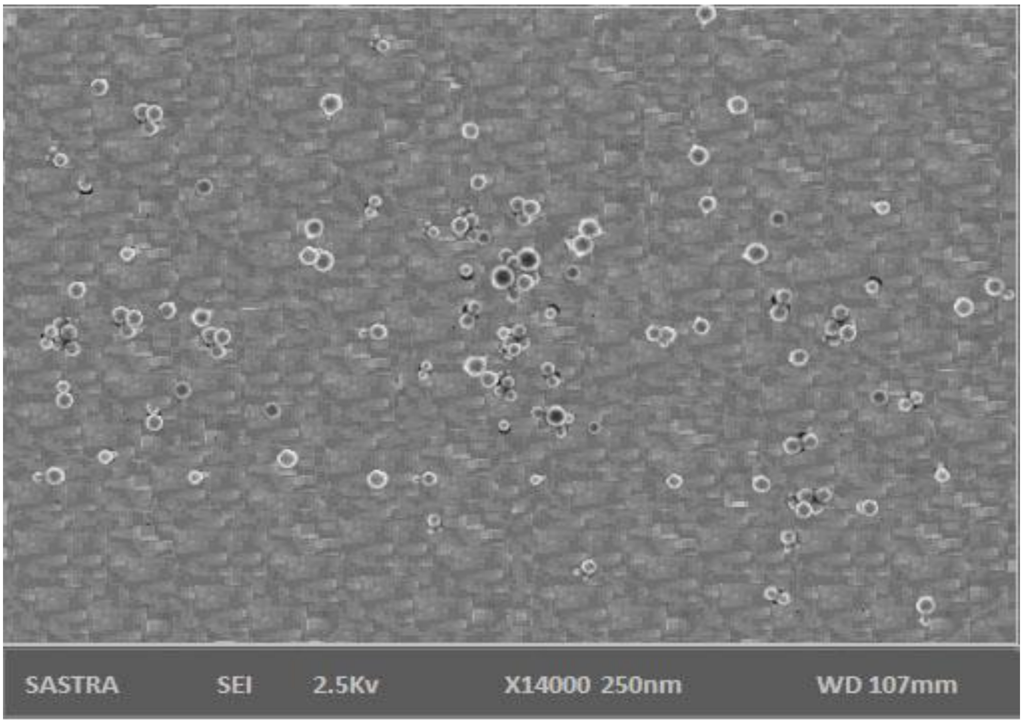


**Figure 38. Scanning electron microscopic (SEM) image of nanoparticles (F17)**

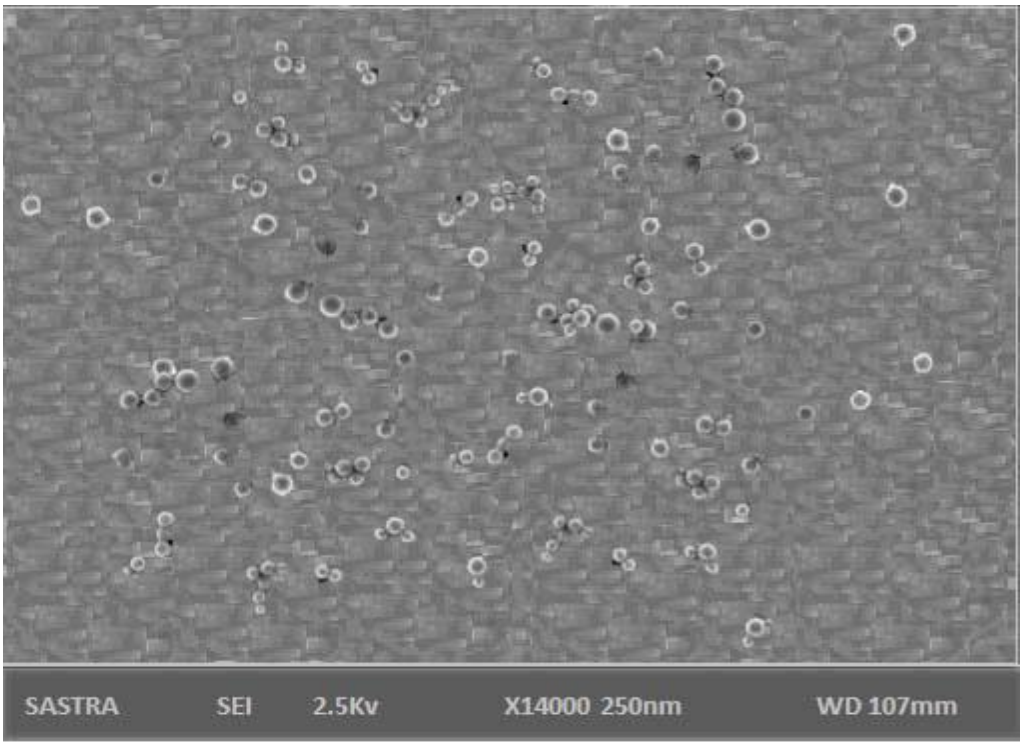


**Figure 39. Scanning electron microscopic (SEM) image of nanoparticles (F18)**

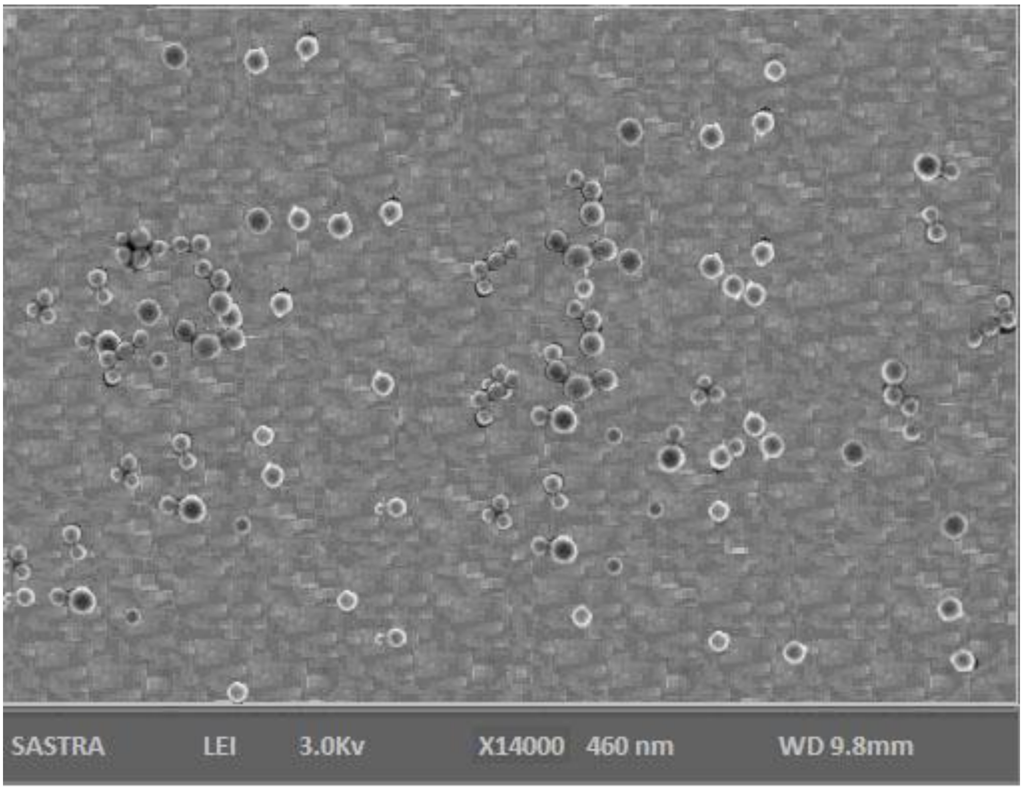


**Figure 40. Scanning electron microscopic (SEM) image of nanoparticles (F19)**

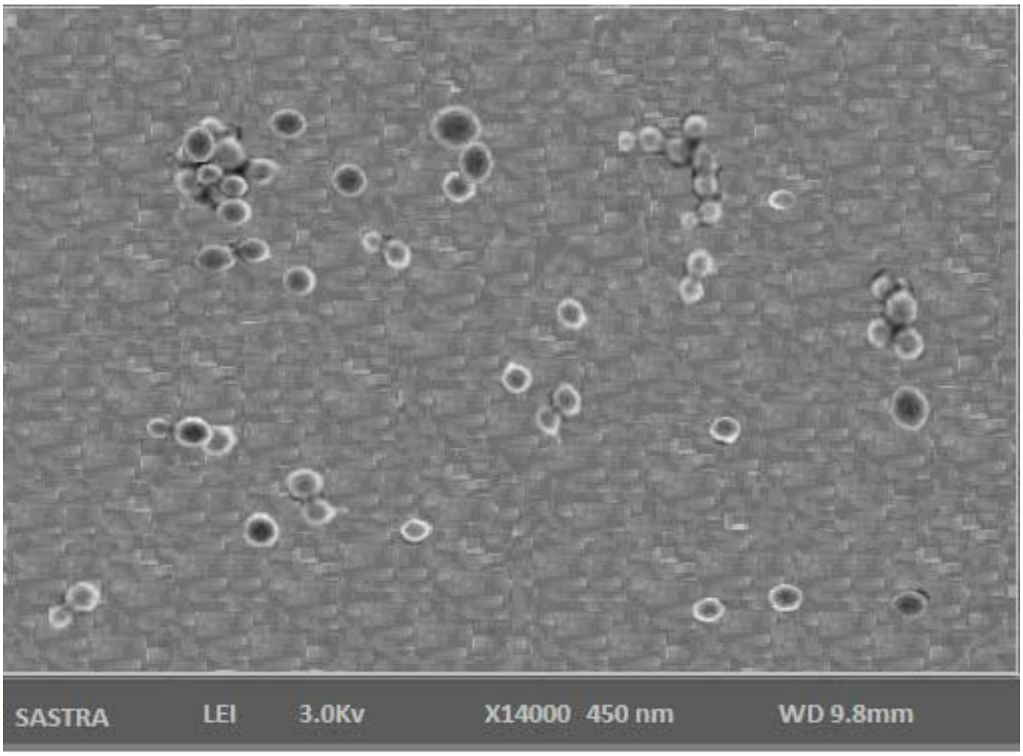


**Figure 41. Scanning electron microscopic (SEM) image of nanoparticles (F20)**

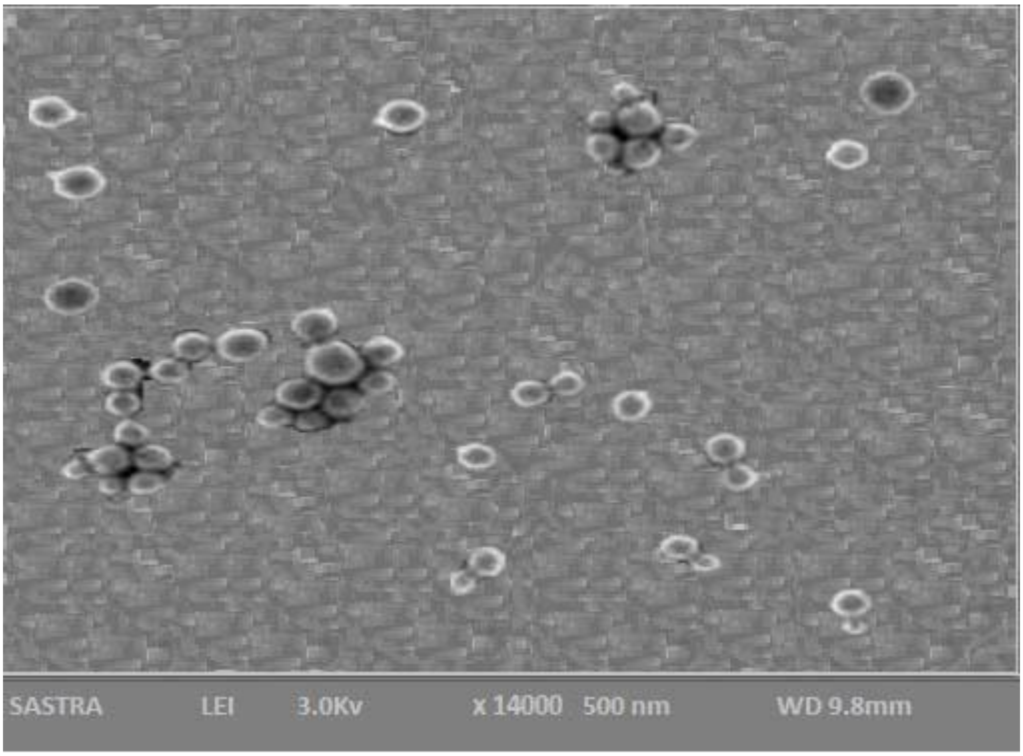


**Figure 42. Scanning electron microscopic (SEM) image of nanoparticles (F21)**

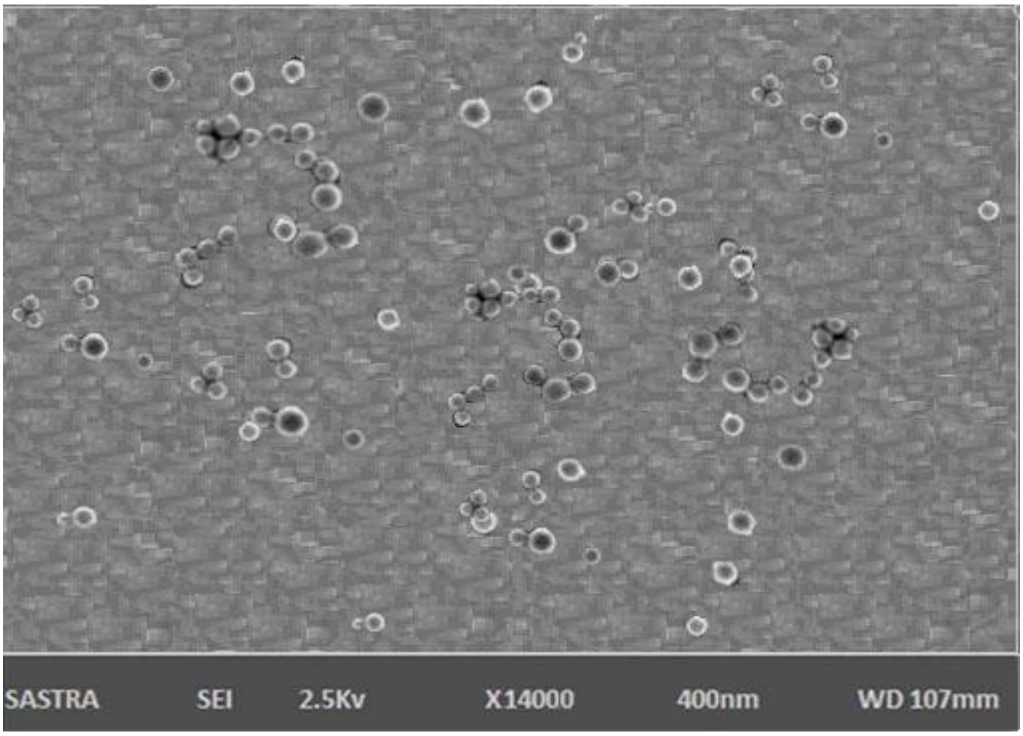


**Figure 43. Scanning electron microscopic (SEM) image of nanoparticles (F22)**

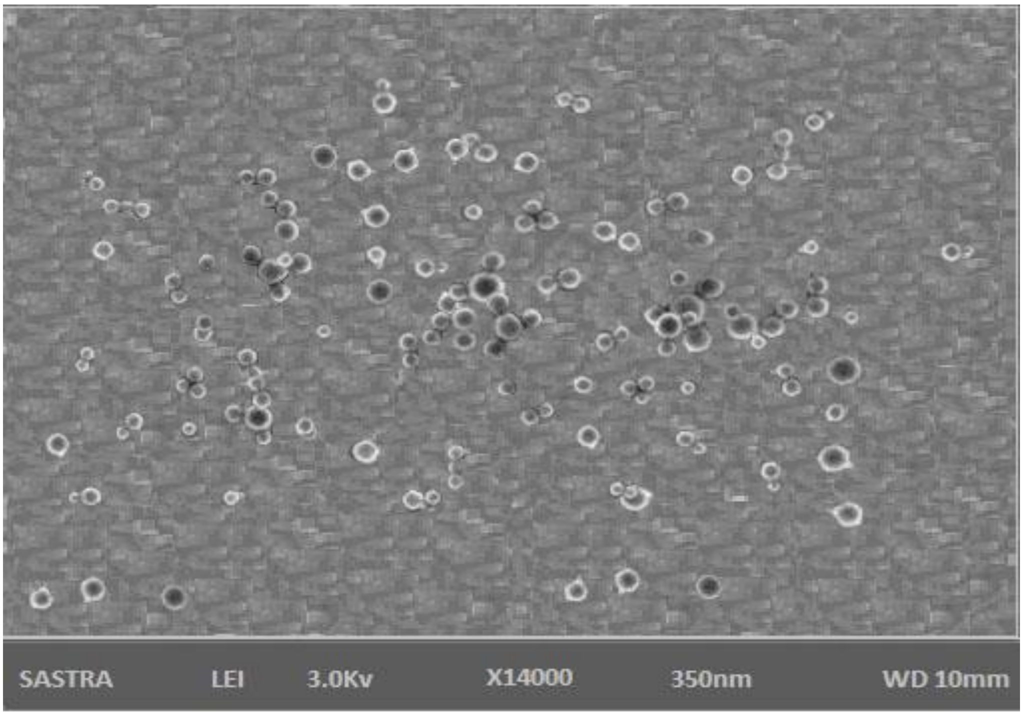


**Figure 44. Scanning electron microscopic (SEM) image of nanoparticles (F23)**

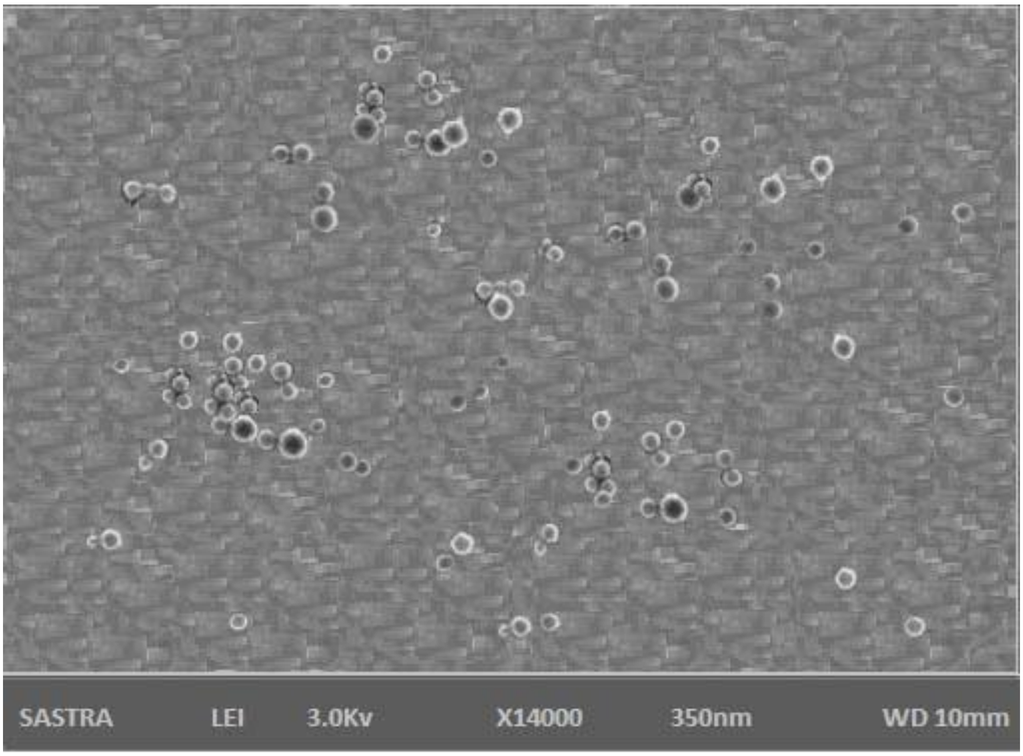


**Figure 45. Scanning electron microscopic (SEM) image of nanoparticles (F24)**

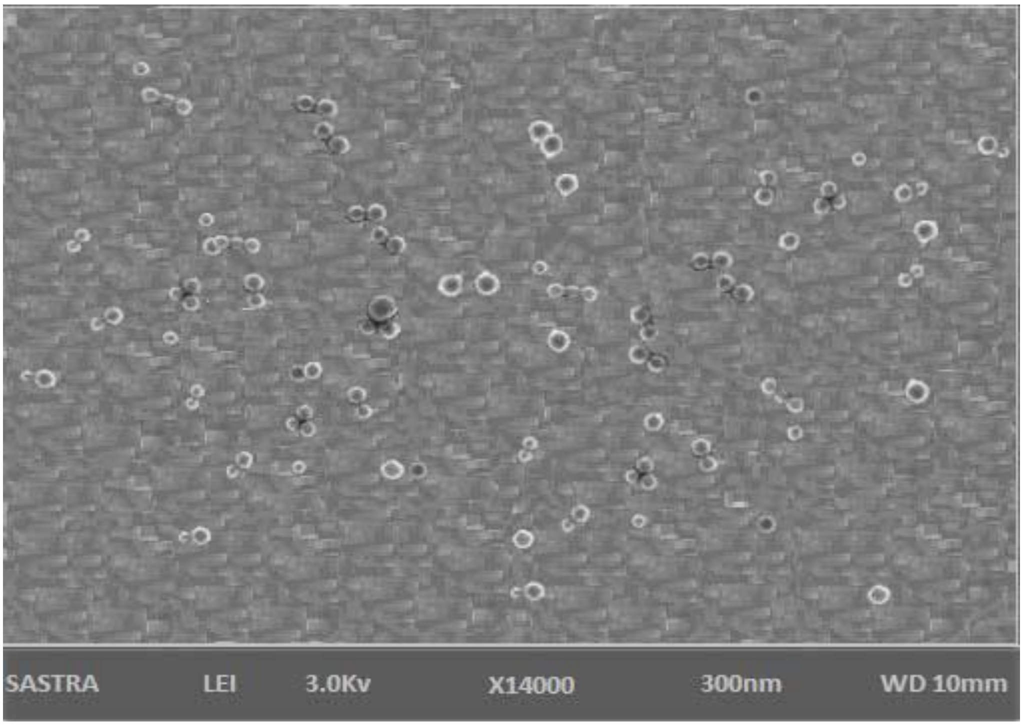


**Figure 46. Scanning electron microscopic (SEM) image of nanoparticles (F25)**

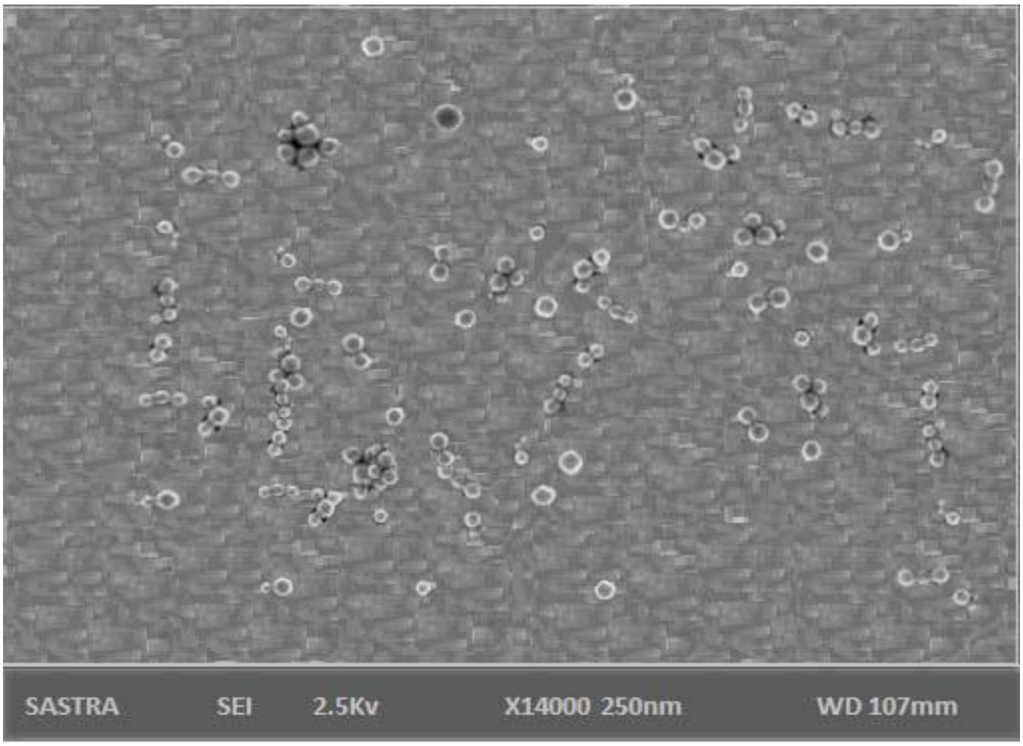


**Figure 47. Scanning electron microscopic (SEM) image of nanoparticles (F26)**

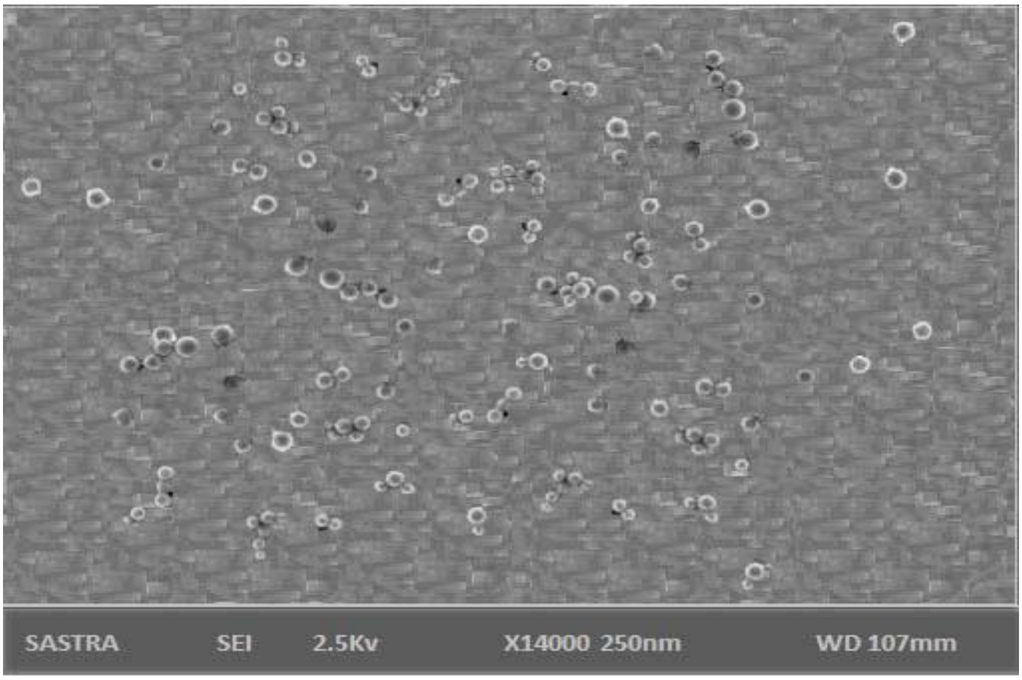


**Figure 48. Scanning electron microscopic (SEM) image of nanoparticles (F27)**

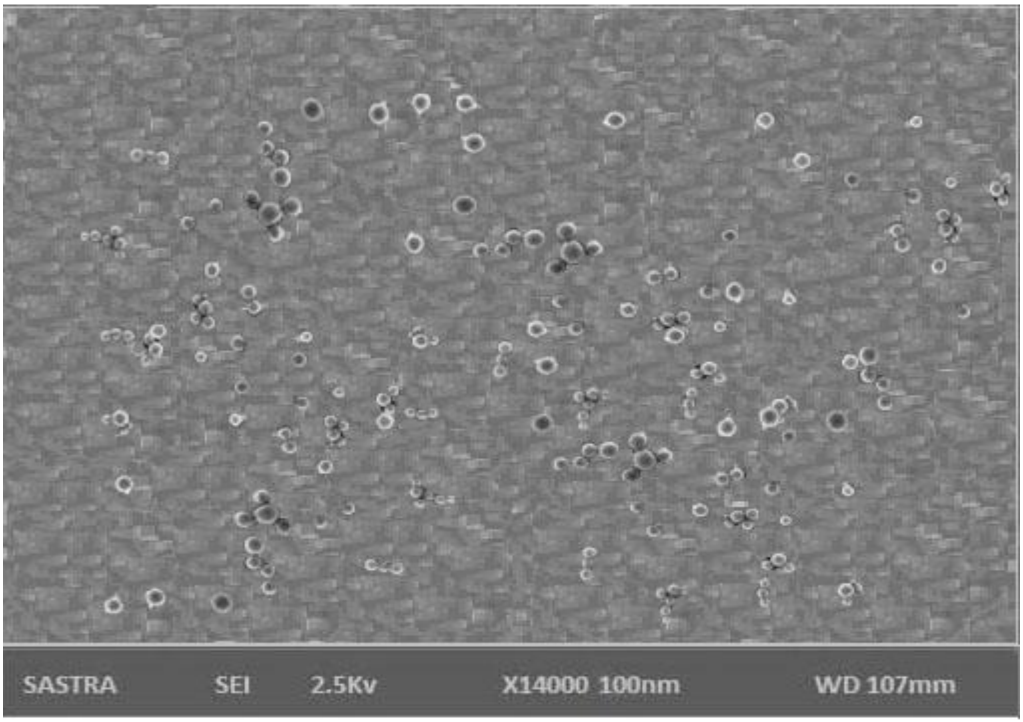


**Figure 49. Scanning electron microscopic (SEM) image of rifampicin-ascorbic acid loaded chitosan nanoparticles (F28)**

**Table 25 Results data of Particle size, Zeta potential, PDI (Mean±SD, n=3)**

| **Formula Code** | **Particle size (nm)** | **Zeta potential (mV)** | **PDI** |
|---|---|---|---|
| F1 | 423±3.43** [a] | +37±0.13* * [a] | 0.429 |
| F2 | 410±2.12* *[a] | +37±0.68* * [a] | 0.431 |
| F3 | 432±1.23* * [a] | +35±0.89* * [a] | 0.495 |
| F4 | 325±2.34* *[a] | +37±0.46* * [a] | 0.302 |
| F5 | 302±1.43* * [a] | +38±0.96* * [a] | 0.285 |
| F6 | 308±3.48* * [a] | +39±0.65* * [a] | 0.301 |
| F7 | 215±2.34* * *[a] | +40±0.85* * *[a] | 0.285 |
| **F8** | **202**±2.76 | **+42**±0.75* * *[a] | **0.225** |
| F9 | 225±3.48* * *[a] | +39±0.45* * *[a] | 0.285 |
| F10 | 460±2.12* * [a] | +38±0.32* * [a] | 0.452 |
| F11 | 442±6.43* * [a] | +37±0.87* * *[a] | 0.441 |
| F12 | 496±2.34* [a] | +32±0.14* [a] | 0.510 |
| F13 | 359±2.12* * [a] | +37±1.01* * [a] | 0.325 |
| F14 | 334±3.48* * [a] | +41±0.98* * *[a] | 0.285 |
| F15 | 334±5.56* * [a] | +39±0.14* * *[a] | 0.323 |
| F16 | 229±4.32* * *[a] | +40±0.78* * *[a] | 0.292 |
| F17 | 212±2.34* * *[a] | +42±0.15* * *[a] | 0.231 |
| F18 | 240±4.67* * *[a] | +39±0.65* * *[a] | 0.273 |
| F19 | 490±3.48* [a] | +38±0.47* * [a] | 0.494 |
| F20 | 453±5.32* * [a] | +37±0.54* * [a] | 0.451 |
| F21 | 513±3.48* [a] | +38±0.36* * [a] | 0.523 |
| F22 | 380±4.21* * [a] | +37±0.58* * *[a] | 0.325 |
| F23 | 345±4.87* * [a] | +41±0.25* * *[a] | 0.292 |
| F24 | 360±2.34* * [a] | +38±0.85* * [a] | 0.335 |
| F25 | 302±2.12* * [a] | +40±0.98* * *[a] | 0.230 |
| F26 | 214±3.48* * *[a] | +42±0.47* * *[a] | 0.235 |
| F27 | 250±5.98* * *[a] | +39±0.25* * *[a] | 0.310 |
| **F28** | **202±5.98** * * *[a] | **+42±0.17** * * *[a] | **0.219** |

***P<0.001, **P<0.01, *P<0.05, [a]Significant difference compared to formulation **F8**

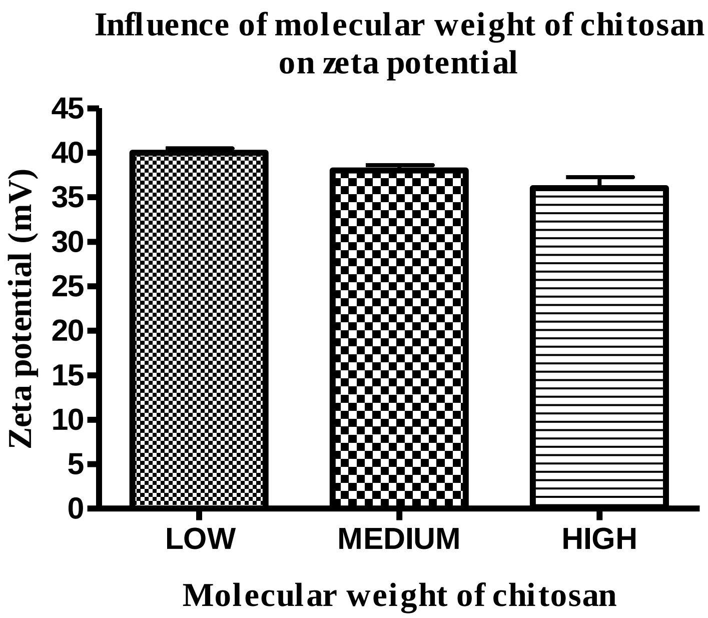


**Figure 50. Influence of molecular weight of chitosan on zeta potential**

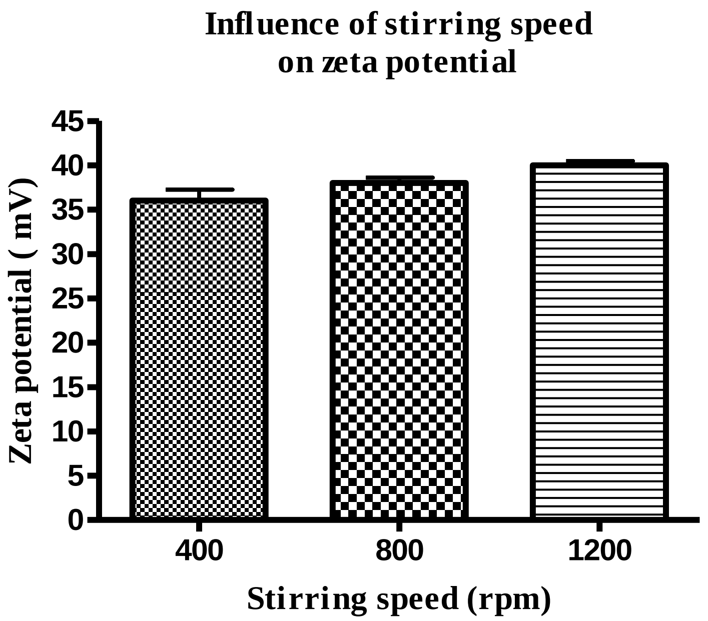


**Figure 51. Influence of stirring speed on zeta potential**

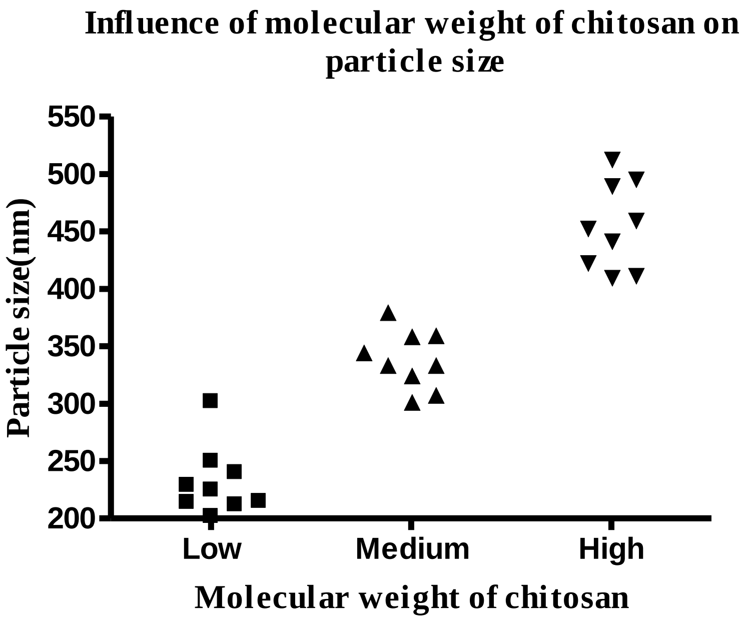


**Figure 52. Influence of molecular weight of chitosan on particle size**

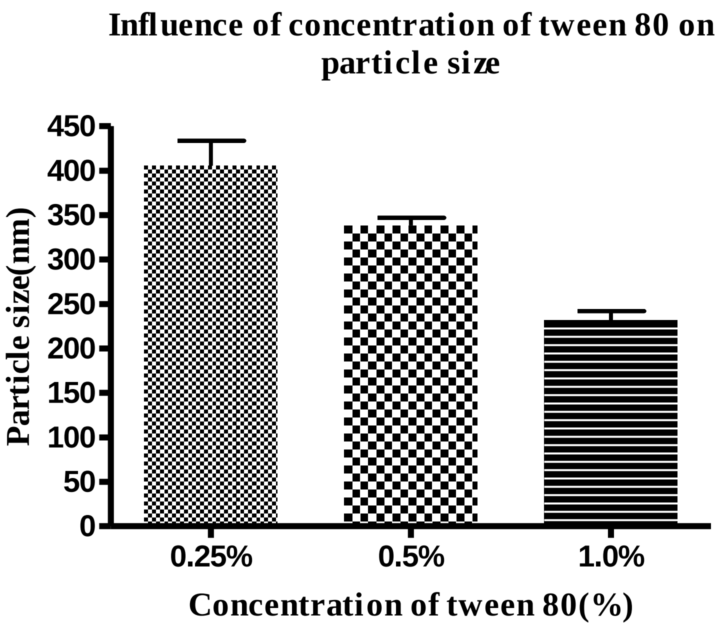


**Figure 53. Influence of concentration of tween 80 on particle size**

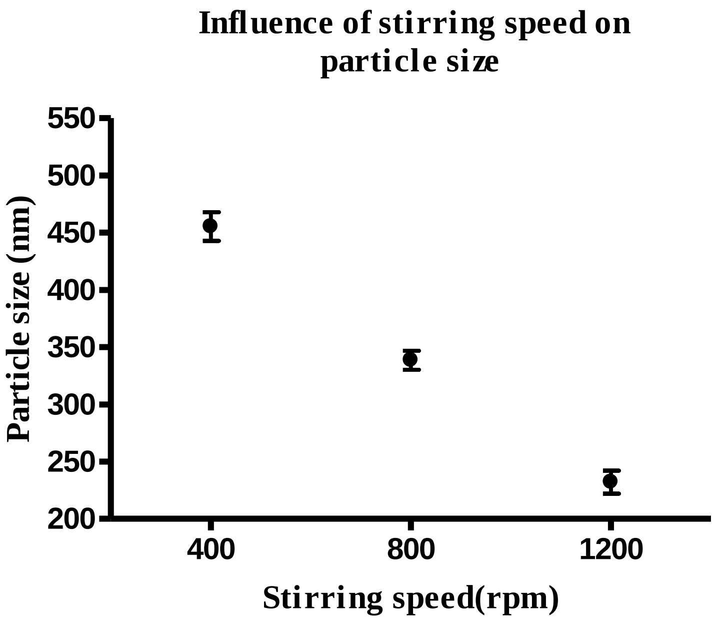


**Figure 54. Influence of stirring speed on particle size**

**Table 26 Data of encapsulation efficiency and loading capacity and (%) diffusion of nanoparticles (Mean±SD, n=3)**

| Formula Code | (%) Encapsulation Efficiency± S.D | (%) Loading Capacity± S.D | (%) Diffusion ± S.D |
|---|---|---|---|
| F1 | 49.82±0.34* * *[a] | 25.96±0.45* * *[a] | 28.75 ±0.33* * [a] |
| F2 | 56.96±0.35* * *[a] | 29.80±0.51* * *[a] | 29.93±0.16* * [a] |
| F3 | 57.85±0.25* * *[a] | 31.76±0.52* * *[a] | 30.06±0.48* * [a] |
| F4 | 62.85±0.32* * *[a] | 35.91±0.32* * *[a] | 30.81±0.11* * [a] |
| F5 | 64.64±0.41* * *[a] | 36.78±0.44* * *[a] | 32.60±0.32* * [a] |
| F6 | 70.19±0.36* * *[a] | 39.69±0.29* * *[a] | 33.01±0.18* * [a] |
| F7 | 77.67±0.24* * *[a] | 43.93±0.34* * *[a] | 40.05±0.15* * *[a] |
| **F8** | **80.71±0.33** | **45.65±0.35*** * *[a] | **45.09±0.33** |
| F9 | 85.00±0.32* * [a] | 48.57±0.62* * *[a] | 44.16±0.25* * *[a] |
| F10 | 50.89±0.15* * *[a] | 27.66±0.35* * *[a] | 26.01±0.62* * [a] |
| F11 | 57.85±0.45* * *[a] | 30.56±0.42* * *[a] | 28.30±0.25* * [a] |
| F12 | 59.10±0.23* * *[a] | 32.13±0.49* * *[a] | 29.63±0.30* * [a] |
| F13 | 66.25±0.31* * *[a] | 35.67±0.43* * *[a] | 29.98±0.24* * [a] |
| F14 | 66.96±0.12* * *[a] | 37.87±0.22* * *[a] | 30.46±0.19* * [a] |
| F15 | 72.50±0.54* * *[a] | 41.01±0.35* * *[a] | 31.09±0.55* * [a] |
| F16 | 78.92±0.22* *[a] | 44.64±0.62* * *[a] | 32.64±0.51* * [a] |
| F17 | 82.14±0.31* * [a] | 46.23±0.32* * *[a] | 35.71±0.22* * [a] |
| F18 | 85.71±0.52* * *[a] | 48.87±0.34* * *[a] | 36.43±0.11* * [a] |
| F19 | 53.92±0.11* * *[a] | 28.49±0.47* * *[a] | 21.39±0.19* [a] |
| F20 | 59.28±0.32* * *[a] | 31.32±0.51* * *[a] | 23.11±0.30* [a] |
| F21 | 61.07±0.26* * *[a] | 33.20±0.19* * *[a] | 23.98±0.29* [a] |
| F22 | 73.92±0.24* * *[a] | 39.42±0.35* * *[a] | 25.15±0.17* **[a] |
| F23 | 68.75±0.31* * *[a] | 38.89±0.21* * *[a] | 28.60±0.20* * [a] |
| F24 | 75.17±0.21* * *[a] | 42.52±0.69* * * [a] | 30.34±0.24* * [a] |
| F25 | 80.00±0.24* [a] | 45.25±0.23* * *[a] | 30.83±0.06* * [a] |
| F26 | 83.57±0.23* * [a] | 47.27±0.22* * *[a] | 33.98±0.16* * [a] |
| F27 | 87.14±0.26* * *[a] | 49.39±0.57* * *[a] | 34.67±0.27* * [a] |
| **F28** | **82.56±0.014*** * [a] | **48.38±0.189*** * *[a] | **49.23±0.17*** * *[a] |

***P<0.001, **P<0.01, *P<0.05, [a]Significant difference compared to formulation **F8**

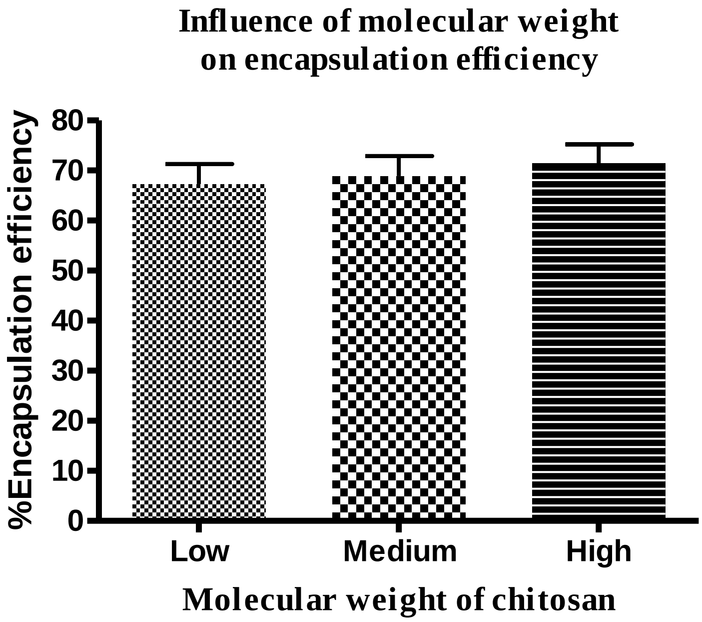


**Figure 55. Influence of molecular weight of chitosan on encapsulation efficiency**

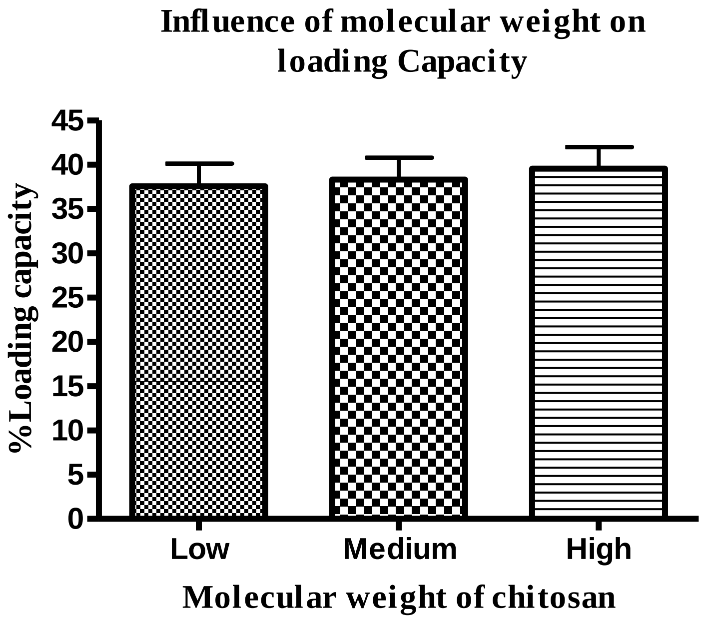


**Figure 56. Influence of molecular weight of chitosan on loading capacity**

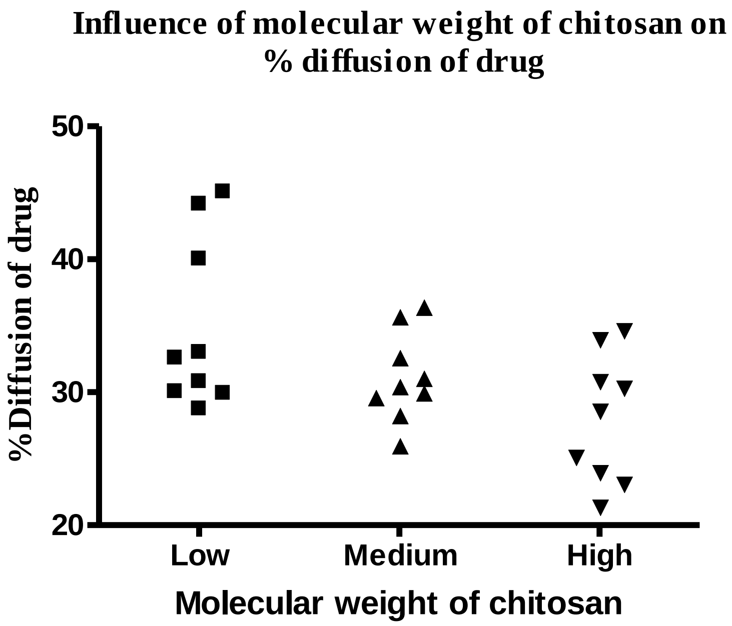


**Figure 57. Influence of molecular weight of chitosan on % diffusion of drug**

***In-vitro* dissolution stability study**

***In-vitro* percentage degradation of rifampicin alone and in combination with isoniazid using ascorbic acid in different concentrations at 475nm.**

**Table 27 Percentage degradation of rifampicin alone at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 33 | 33.2 | 33.1 | 33.1±0.10 |
| 30 | 37 | 38.8 | 37.6 | 37.8±0.916 |
| 45 | 40 | 39.2 | 36.3 | 38.5±1.94 |
| 60 | 44 | 43.6 | 40.6 | 42.7±1.85 |

**Table 28 Percentage degradation of rifampicin in the presence of isoniazid at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 54.8 | 56.3 | 56.6 | 55.9±0.96 |
| 30 | 61.2 | 61.4 | 62 | 61.5±0.41 |
| 45 | 74 | 73.8 | 74.2 | 74±0.207 |
| 60 | 78.5 | 78.6 | 79.4 | 78.8±0.49 |

**Table 29 Percentage degradation of rifampicin in the presence of ascorbic acid (125mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 23.4 | 24.2 | 23.8 | 23.8±0.40 |
| 30 | 29.2 | 28.6 | 28.6 | 28.8±0.34 |
| 45 | 32.3 | 32.2 | 31.9 | 32.1±0.16 |
| 60 | 35 | 34.8 | 35.2 | 35.0±0.20 |

**Table 30 Percentage degradation of rifampicin in the presence of ascorbic acid (250mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 15.6 | 15.3 | 14.9 | 15.2±0.35 |
| 30 | 19.5 | 19.4 | 19.6 | 19.5±0.10 |
| 45 | 29.2 | 29.4 | 29.0 | 29.2±0.20 |
| 60 | 32.2 | 32.5 | 32.9 | 32.5±0.35 |

**Table 31 Percentage degradation of rifampicin in the presence of ascorbic acid (500mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 14.9 | 14.8 | 15.2 | 14.9±0.20 |
| 30 | 16.2 | 15.8 | 15.6 | 15.8±0.30 |
| 45 | 18.5 | 18.8 | 18.6 | 18.6±0.15 |
| 60 | 20.4 | 20.2 | 20.7 | 20.40±0.25 |

**Table 32 Percentage degradation of rifampicin in the presence of ascorbic acid (1000mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 15.3 | 15 | 14.9 | 15.06±0.20 |
| 30 | 16.2 | 16.6 | 16.5 | 16.4±0.20 |
| 45 | 18.8 | 18.8 | 17.2 | 18.9±0.23 |
| 60 | 21.0 | 20.8 | 20.6 | 20.8±0.20 |

**Table 33 Percentage degradation of rifampicin in the presence of isoniazid and ascorbic acid (125mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 35.9 | 35.7 | 35.8 | 35.8±0.10 |
| 30 | 40 | 40.8 | 40.6 | 40.4±0.41 |
| 45 | 48.8 | 49.5 | 49.2 | 49.1±0.35 |
| 60 | 58.6 | 58.4 | 58.2 | 58.4±0.20 |

**Table 34 Percentage degradation of rifampicin in the presence of isoniazid and ascorbic acid (250mg) at pH1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 29 | 30 | 28.7 | 29.2±0.68 |
| 30 | 40 | 38.9 | 38 | 38.9±1.00 |
| 45 | 44 | 43.3 | 44.2 | 43.8±0.47 |
| 60 | 52 | 53.7 | 53.3 | 53.0±0.88 |

**Table 35 Percentage degradation of rifampicin in the presence of isoniazid and ascorbic acid (500mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 26 | 25.7 | 25 | 25.5±0.51 |
| 30 | 37 | 36.9 | 37.3 | 37.0±0.20 |
| 45 | 41 | 40.7 | 40 | 40.5±0.51 |
| 60 | 45 | 46.3 | 45.1 | 45.4±0.72 |

**Table 36 Percentage degradation of rifampicin in the presence of isoniazid and ascorbic acid (1000mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 24.1 | 25.2 | 25.2 | 24.8±0.63 |
| 30 | 37.2 | 36.9 | 37.3 | 37.1±0.20 |
| 45 | 40.1 | 40.7 | 40.1 | 40.3±0.34 |
| 60 | 44.3 | 46.3 | 44.3 | 44.9±1.15 |

**Table 37 Percentage degradation of rifampicin nanoparticles at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 14.9 | 15.5 | 15.2 | 15.2±0.30 |
| 30 | 19.4 | 19.3 | 20 | 19.5±0.37 |
| 45 | 29.3 | 29 | 29.4 | 29.2±0.20 |
| 60 | 31.9 | 32.4 | 32.3 | 32.15±0.26 |

**Table 38 Percentage degradation of rifampicin nanoparticles + isoniazid at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 29.5 | 29.6 | 29.2 | 29.4±0.20 |
| 30 | 33.8 | 34.6 | 34.4 | 34.2±0.41 |
| 45 | 43 | 42.8 | 43.5 | 43.0±0.36 |
| 60 | 56.6 | 56.7 | 57 | 56.76±0.20 |

**Table 39 Percentage degradation of rifampicin - ascorbic acid (500mg) nanoparticles at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 14.9 | 15.5 | 15.2 | 15.2±0.30 |
| 30 | 17.5 | 17.8 | 17.6 | 17.6±0.15 |
| 45 | 21.7 | 22 | 21.7 | 21.8±0.17 |
| 60 | 26 | 25.6 | 26.2 | 25.9±1.30 |

**Table 40 Percentage degradation of rifampicin - ascorbic acid (500mg) nanoparticles + isoniazid at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 12.9 | 13.5 | 13.4 | 13.2±0.32 |
| 30 | 16.7 | 17 | 16.8 | 16.8±0.15 |
| 45 | 18.2 | 17.9 | 18 | 18.0±0.15 |
| 60 | 20.5 | 21.2 | 20.8 | 20.8±0.35 |

**Table 41 Percentage rifampicin degradation with isoniazid and ascorbic acid at pH 1.2 buffer (mean±SD, n=3)**

| Time (min) | Rifampicin alone | Rifampicin +isoniazid | Rifampicin - ascorbic acid 125mg | Rifampicin- ascorbic acid 250mg | Rifampicin- ascorbic acid 500mg | Rifampicin- ascorbic acid 1000mg |
|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 33.1±0.10 | 55.9±0.96 | 23.8±0.40 | 15.2±0.35 | 14.9±0.20 | 15.06±0.20 |
| 30 | 37.8±0.92 | 61.5±0.41 | 28.8±0.34 | 19.5±0.10 | 15.8±0.30 | 16.4±0.20 |
| 45 | 38.5±1.94 | 74±0.207 | 32.1±0.16 | 29.2±0.20 | 18.6±0.15 | 18.9±0.23 |
| 60 | 42.7±1.85 | 78.8±0.49 | 35.0±0.20 | 32.5±0.35 | 20.40±0.25 | 20.8±0.20 |

**Table 42 Percentage rifampicin degradation with isoniazid + ascorbic acid at pH 1.2 buffer (mean±SD, n=3)**

| Time (min) | Rifampicin alone | Rifampicin +isoniazid | Rifampicin -ascorbic acid 125mg +isoniazid | Rifampicin - ascorbic acid 250mg +isoniazid | Rifampicin - ascorbic acid 500mg +isoniazid | Rifampicin -ascorbic acid 1000mg +isoniazid |
|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 33.1±0.10 | 55.9±0.96 | 35.8±0.10 | 29.2±0.68 | 25.5±0.51 | 24.8±0.63 |
| 30 | 37.8±0.92 | 61.5±0.41 | 40.4±0.41 | 38.9±1.00 | 37.0±0.20 | 37.1±0.20 |
| 45 | 38.5±1.94 | 74±0.207 | 49.1±0.35 | 43.8±0.47 | 40.5±0.51 | 40.3±0.34 |
| 60 | 42.7±1.85 | 78.8±0.49 | 58.4±0.20 | 53.0±0.88 | 45.4±0.72 | 44.9±1.15 |

**Table 43 Cumulative data on percentage rifampicin degradation from nanoparticles in the presence of isoniazid and ascorbic acid at 475nm in pH 1.2 buffer（mean±SD, n=3）**

| Time (min) | Rifampicin | Rifampicin + isoniazid | Rifampicin -ascorbic acid (500mg) | Rifampicin ascorbic acid + isoniazid | Rifampicin nps | Rifampicin - ascorbic acid nps | Rifampicin nps + isoniazid | Rifampicin - ascorbic acid nps +isoniazid |
|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 33.1±0.10 | 55.9±0.96 | 14.9±0.20 | 25.5±0.51 | 15.2±0.30 * * *[a] | 29.4±0.20* * *[b] | 15.8±0.39* * *[c] | 13.2±0.32* * *[d] |
| 30 | 37.8±0.92 | 61.5±0.41 | 15.8±0.30 | 37.0±0.20 | 19.5±0.37* * *[a] | 34.2±0.41* * *[b] | 17.6±0.15* * *[c] | 16.8±0.15* * *[d] |
| 45 | 38.5±1.94 | 74±0.207 | 18.6±0.15 | 40.5±0.51 | 29.2±0.20* * *[a] | 43.0±0.36* * *[b] | 21.8±0.17* * *[c] | 18.0±0.15* * *[d] |
| 60 | 42.7±1.85 | 78.8±0.49 | 20.40±0.25 | 45.4±0.72 | 32.2±0.26* * *[a] | 56.8±0.20* * *[b] | 25.9±1.30* * *[c] | 20.8±0.35* * *[d] |

***P<0.001, **P<0.01, *P<0.05

[a]Significant difference compared to Rifampicin alone

[b]Significant difference compared to Rifampicin- ascorbic acid (500mg)

[c]Significant difference compared to Rifampicin+ isoniazid

[d]Significant difference compared to Rifampicin- ascorbic acid (500mg) + isoniazid

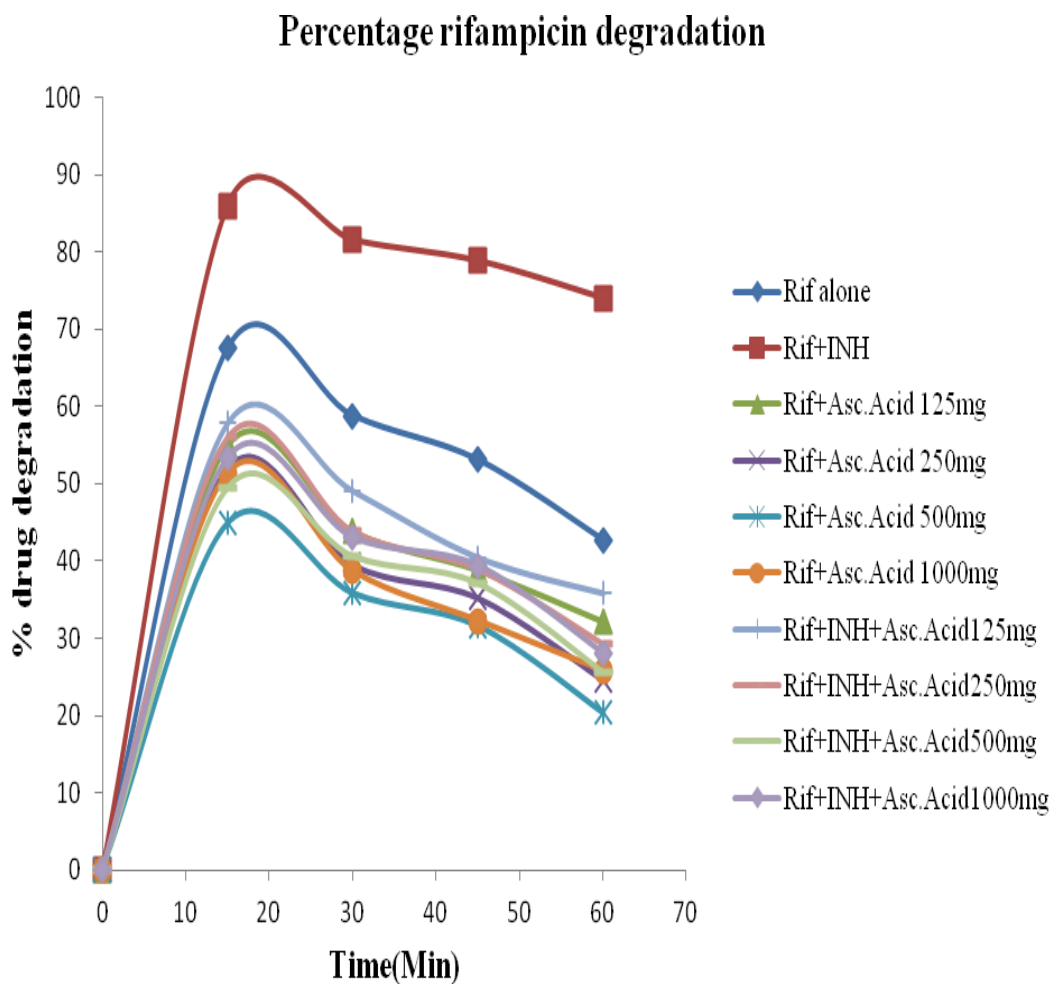


**Figure 58. Percentage rifampicin degradation**

***In-vitro*-diffusion**

***In-vitro* percentage release of rifampicin alone and in combination with isoniazid   using ascorbic acid in different concentrations at pH 1.2 buffer**

**Table 44 Percentage release of rifampicin alone at pH 1.2**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 32.2 | 32.4 | 32.6 | 32.4±0.20 |
| 30 | 41.3 | 41 | 41.5 | 41.2±0.25 |
| 45 | 47 | 47.2 | 46.7 | 46.9±0.25 |
| 60 | 48.6 | 48.9 | 48.5 | 48.6±0.20 |

**Table 45 Percentage release of rifampicin in the presence of isoniazid**

**at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 10 | 10.5 | 10.3 | 10.23±0.25 |
| 30 | 12.6 | 12.4 | 13 | 12.68±0.30 |
| 45 | 14.4 | 14.3 | 14.4 | 14.37±0.05 |
| 60 | 18 | 18.1 | 18.3 | 18.1±0.15 |

**Table 46 Percentage release of rifampicin in the presence of ascorbic acid (500mg) at pH 1.2 buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 45.0 | 45.2 | 44.8 | 45±0.20 |
| 30 | 56.6 | 56.2 | 56.4 | 56.4±0.20 |
| 45 | 61.0 | 61.5 | 61 | 61.±0.28 |
| 60 | 67.4 | 67.2 | 67 | 67.2±0.20 |

**Table 47 Percentage release of rifampicin in the presence of ascorbic acid and isoniazid buffer**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 40 | 40.2 | 40.4 | 40.2±0.20 |
| 30 | 42 | 42.3 | 42.3 | 42.1±0.15 |
| 45 | 54 | 54.2 | 53.7 | 53.9±0.25 |
| 60 | 60 | 60.2 | 60.0 | 60.04±0.11 |

**Table 48 Percentage release of rifampicin from nanoparticles**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 47.8 | 48.3 | 48 | 48±0.251 |
| 30 | 60.6 | 60.2 | 60.8 | 60.5±0.30 |
| 45 | 65 | 64.6 | 65 | 64.8±0.23 |
| 60 | 66.6 | 66.4 | 65.8 | 66.23±0.41 |

**Table 49 Percentage rifampicin release of rifampicin nanoparticles with isoniazid**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 18.5 | 18.8 | 18.4 | 18.5±0.20 |
| 30 | 22.2 | 22.4 | 21.8 | 22.13±0.30 |
| 45 | 34.3 | 33.7 | 34.2 | 34.0±0.32 |
| 60 | 38.6 | 39 | 38.8 | 38.83±0.20 |

**Table 50 Percentage release of rifampicin from rifampicin - ascorbic acid nanoparticles**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 55.0 | 55.4 | 54.8 | 55.0±0.30 |
| 30 | 64.1 | 64.4 | 64.0 | 64.1±0.20 |
| 45 | 68.3 | 68.5 | 68.4 | 68.4±0.10 |
| 60 | 73.8 | 74.0 | 74.4 | 74.0±1.30 |

**Table 51 Percentage release of rifampicin from rifampicin - ascorbic acid nanoparticles in the presence of isoniazid**

| Time(min) | Trial 1 | Trial 2 | Trial 3 | Mean±SD |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 15 | 41.8 | 42.4 | 42 | 42.03±0.30 |
| 30 | 58.7 | 58.5 | 58.2 | 58.46±0.25 |
| 45 | 63.8 | 64.2 | 63.9 | 63.9±0.20 |
| 60 | 69 | 68.6 | 69.2 | 68.9±0.30 |

**Table 52 Cumulative data on percentage rifampicin release from nanoparticles in the presence of isoniazid and ascorbic acid at 475nm in pH 1.2 buffer (mean±SD, n=3)**

| Time (min) | Rifampicin alone | Rifampicin + isoniazid | Rifampicin -ascorbic acid | Rifampicin ascorbic acid + isoniazid | Rifampicin nps | Rifampicin nps + isoniazid | Rifampicin - ascorbic acid nps | Rifampicin - ascorbic acid nps +isoniazid |
|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 32.4±0.20 | 10.2±0.25 | 45.0±0.20 | 40.2±0.20 | 48.0±0.25* * *[a] | 18.5±0.20* * *[b] | 55.1±0.30* * *[c] | 42.0±0.30* * *[d] |
| 30 | 41.2±0.25 | 12.7±0.30 | 56.4±0.20 | 42.1±0.15 | 60.5±0.30* * *[a] | 22.1±0.30* * *[b] | 64.1±0.20* * *[c] | 58.5±0.25* * *[d] |
| 45 | 46.9±0.25 | 14.4±0.05 | 61.0±0.28 | 53.9±0.25 | 64.8±0.23* * *[a] | 34.0±0.32* * *[b] | 68.4±0.10* * *[c] | 63.9±0.20* * *[d] |
| 60 | 48.6±0.20 | 18.1±0.15 | 67.2±0.20 | 60.0±0.11 | 66.2±0.41* * *[a] | 38.8±0.20* * *[b] | 74.0±1.30* * *[c] | 68.9±0.30* * *[d] |

***P<0.001, **P<0.01, *P<0.05,

[a]Significant difference compared to Rifampicin alone

[b]Significant difference compared to Rifampicin- ascorbic acid (500mg)

[c]Significant difference compared to Rifampicin+ isoniazid

[d]Significant difference compared to Rifampicin- ascorbic acid (500mg) + isoniazid

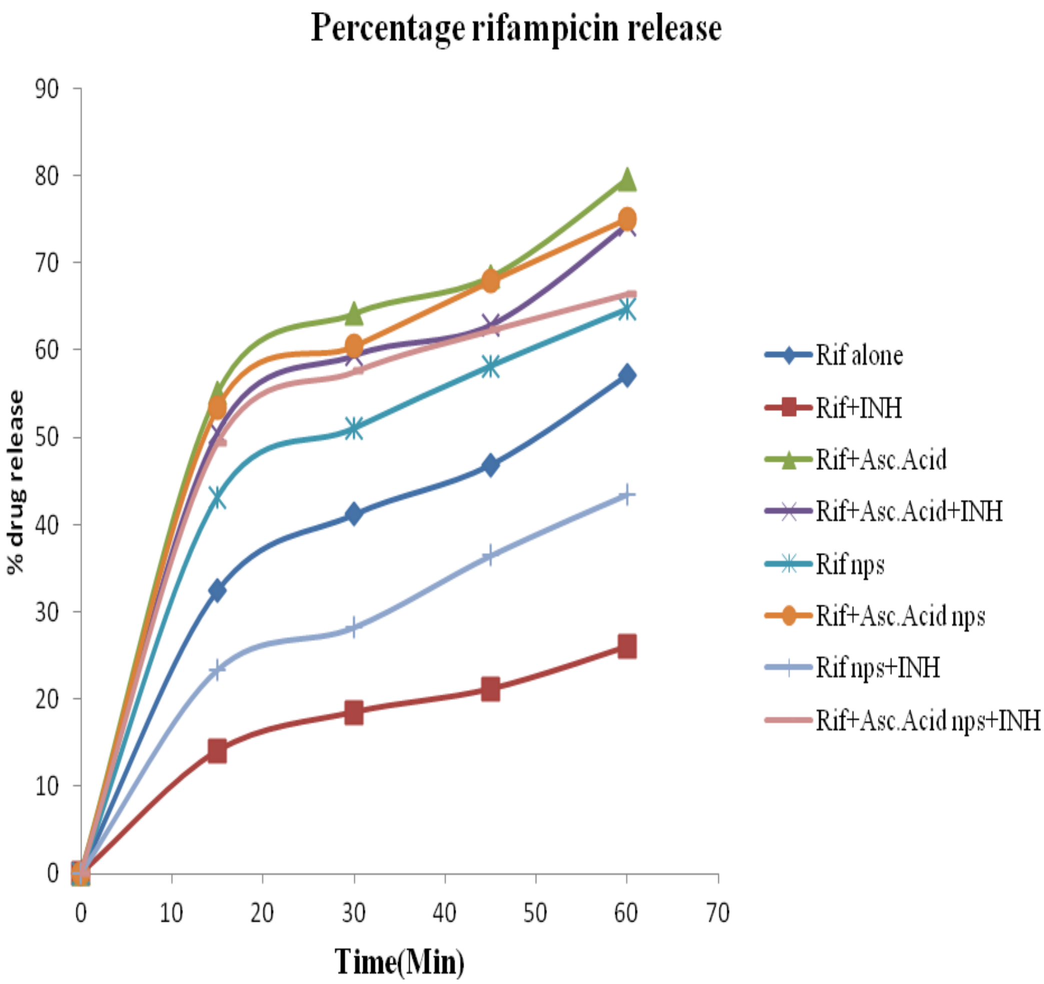


**Figure 59. Percentage rifampicin release**

**Plasma drug concentration at various time intervals by HPLC method**

**Table 53 Group I - Rifampicin alone (n=6, mean±SD)**

| **Time(hr)** | **Concentration(μg/ml)** | | | | | | **Mean± S.D** |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | |
| 0.5 | 3.9 | 3.5 | 3.3 | 4.0 | 4.1 | 3.7 | 3.80±0.18 |
| 1 | 4.0 | 4.3 | 4.1 | 4.2 | 4.1 | 4.4 | 4.20±0.13 |
| 2 | 4.8 | 5.1 | 4.7 | 5.0 | 5.3 | 5.2 | 5.10±0.20 |
| 4 | 5.6 | 4.9 | 6.0 | 6.2 | 7.0 | 5.7 | 5.91±0.12 |
| 6 | 3.7 | 3.8 | 3.3 | 3.4 | 3.3 | 3.2 | 3.40±0.16 |
| 9 | 0.9 | 0.8 | 1.2 | 1.0 | 0.7 | 1.1 | 1.0±0.01 |
| 12 | - | - | - | - | - | - | - |

**HPLC Chromatograms**

**GROUP I- Rifampicin alone**

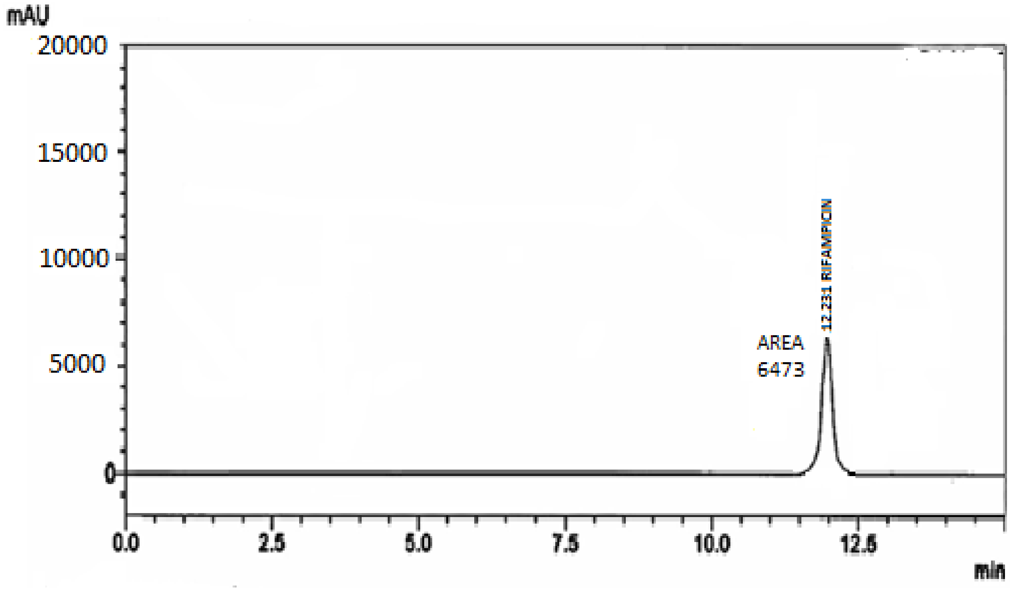


**Figure 60 (a). 0.5 hr**

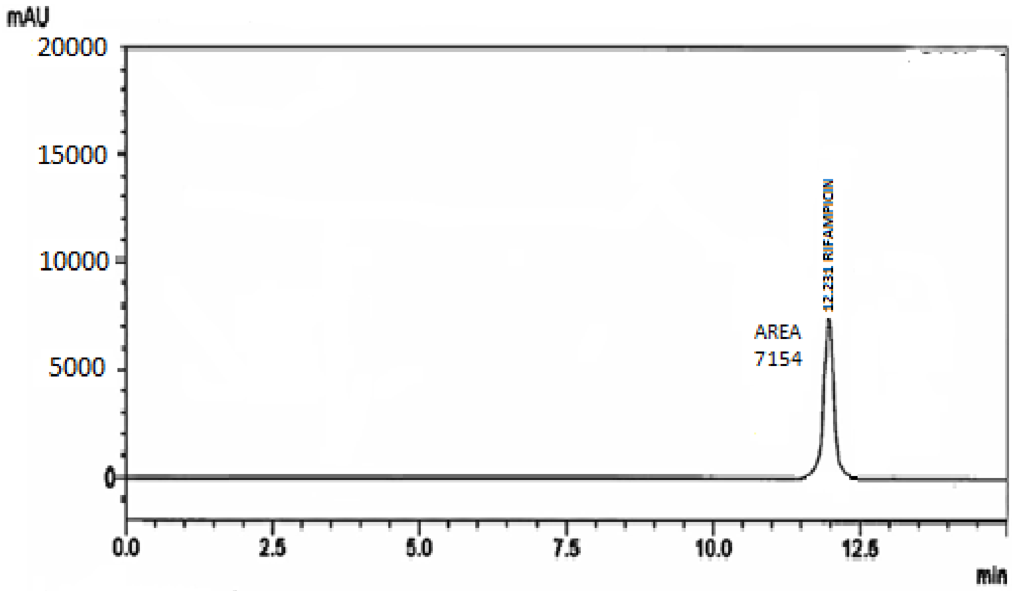


**Figure 60(b). 1st hr**

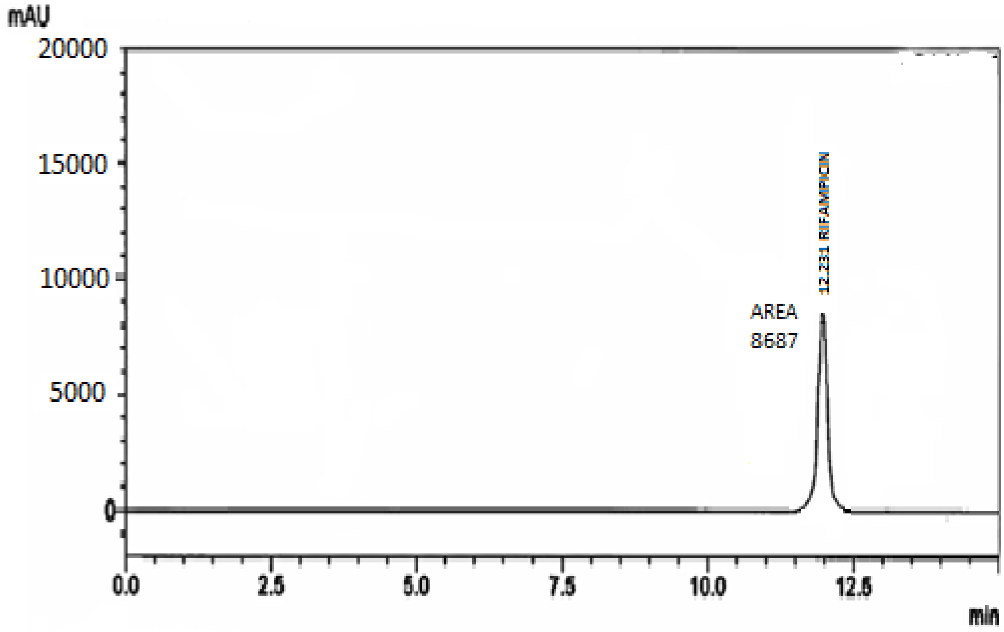


**Figure 60(c). 2nd hr**

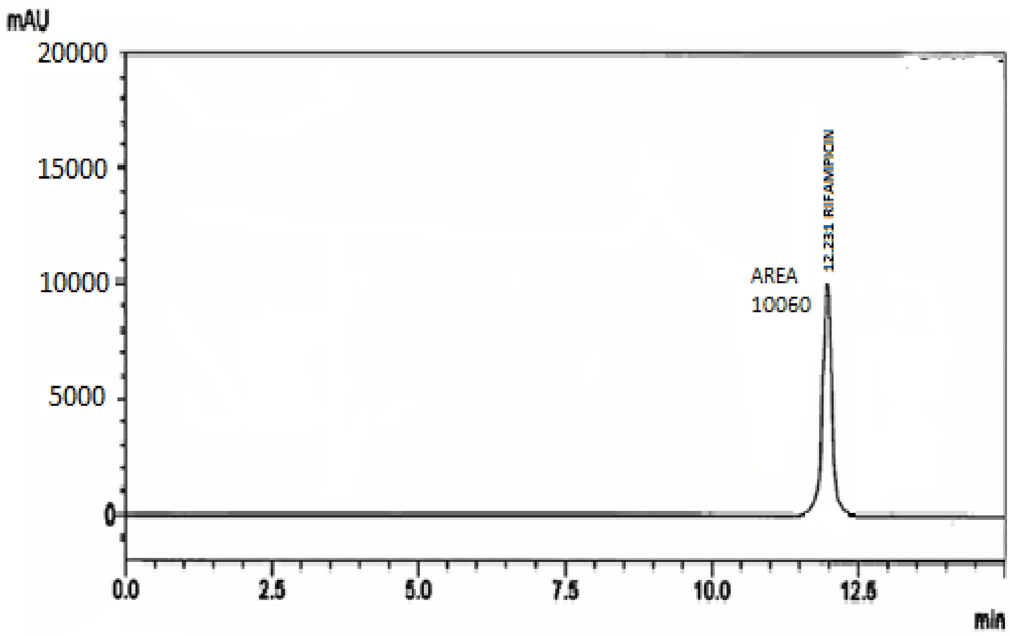


**Figure 60(d). 4th hr**

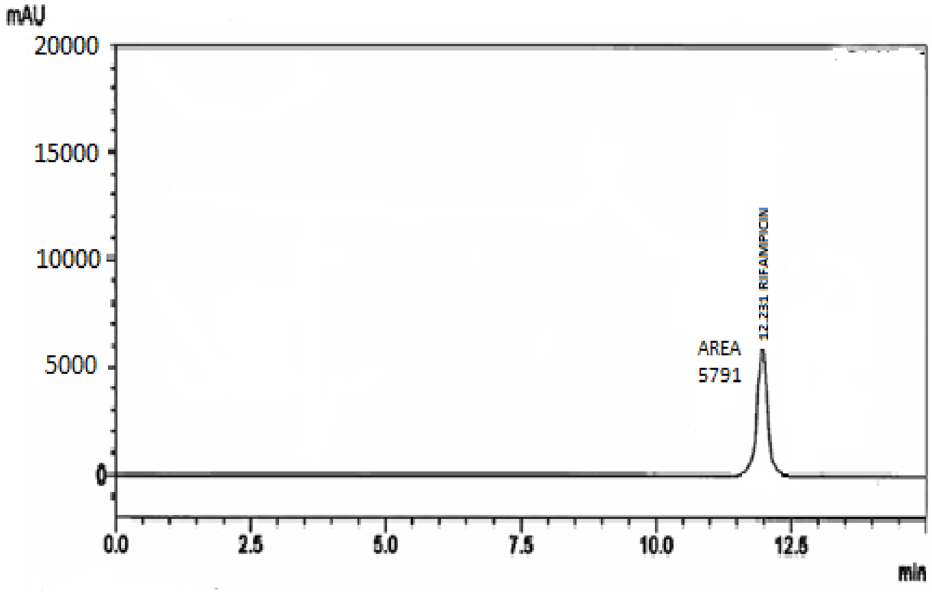


**Figure 60(e). 6th hr**

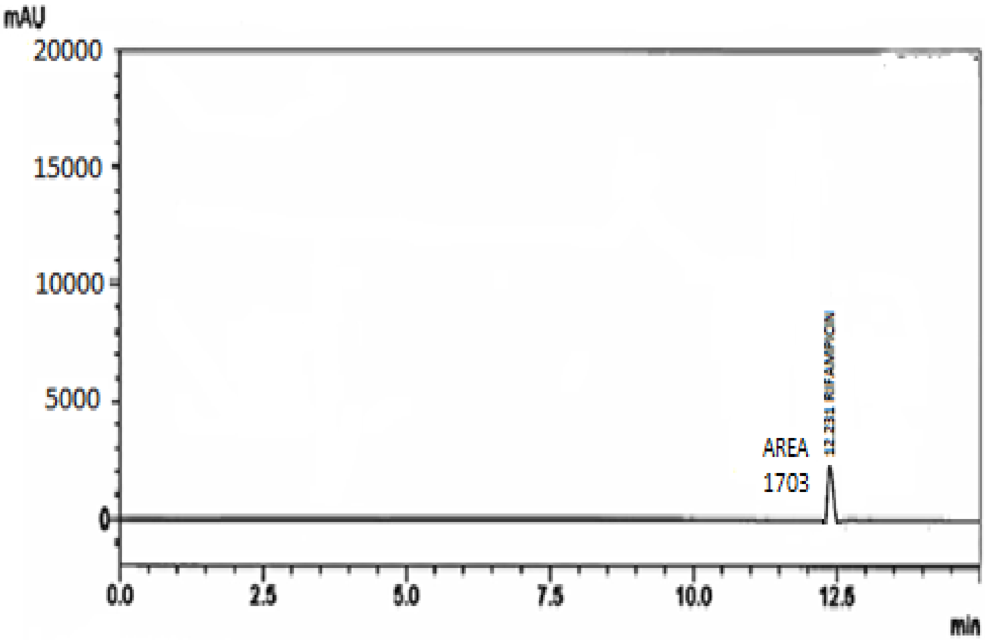


**Figure 60(f). 9th hr**

**Table 54 Group-II - Rifampicin + isoniazid (n=6, mean±SD)**

| Time(hr) | Concentration(µg/ml) | | | | | | Mean± S.D |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| 0.5 | 1.9 | 2.3 | 2.6 | 2.0 | 2.1 | 2.8 | 2.2±0.202 |
| 1 | 2.9 | 3.0 | 2.9 | 3.0 | 2.8 | 2.9 | 2.9±0.1155 |
| 2 | 3.1 | 3.3 | 3.4 | 3.2 | 3.5 | 3.4 | 3.2±0.115 |
| 4 | 3.9 | 4.0 | 4.1 | 3.7 | 4.3 | 4.1 | 4.09±0.046 |
| 6 | 2.9 | 3.0 | 2.8 | 2.6 | 3.1 | 2.8 | 2.9±0.054 |
| 9 | - | - | - | - | - | - | - |
| 12 | - | - | - | - | - | - | - |

**HPLC Chromatograms**

**Group-II - Rifampicin + isoniazid**

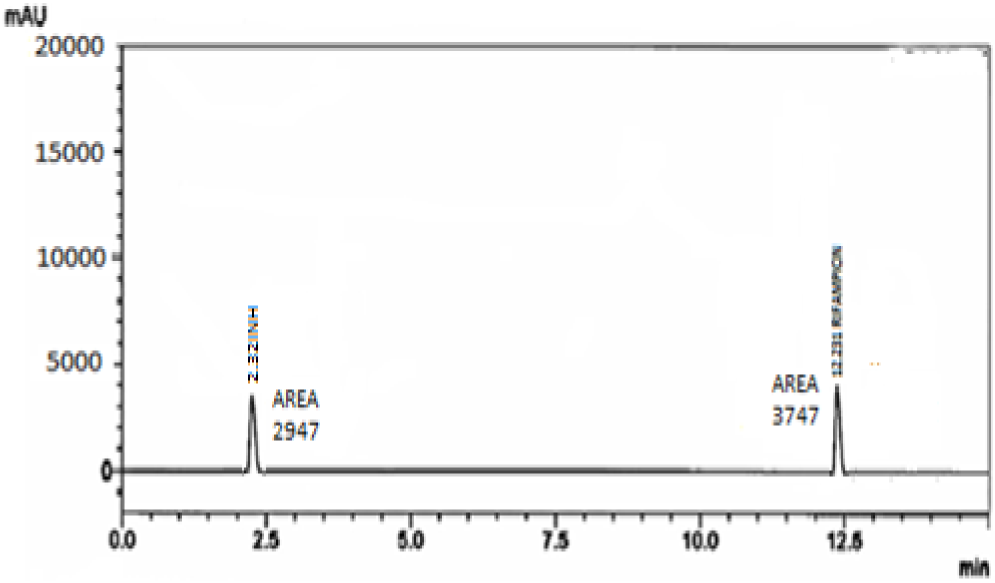


**Figure 61(a). 0.5hr**

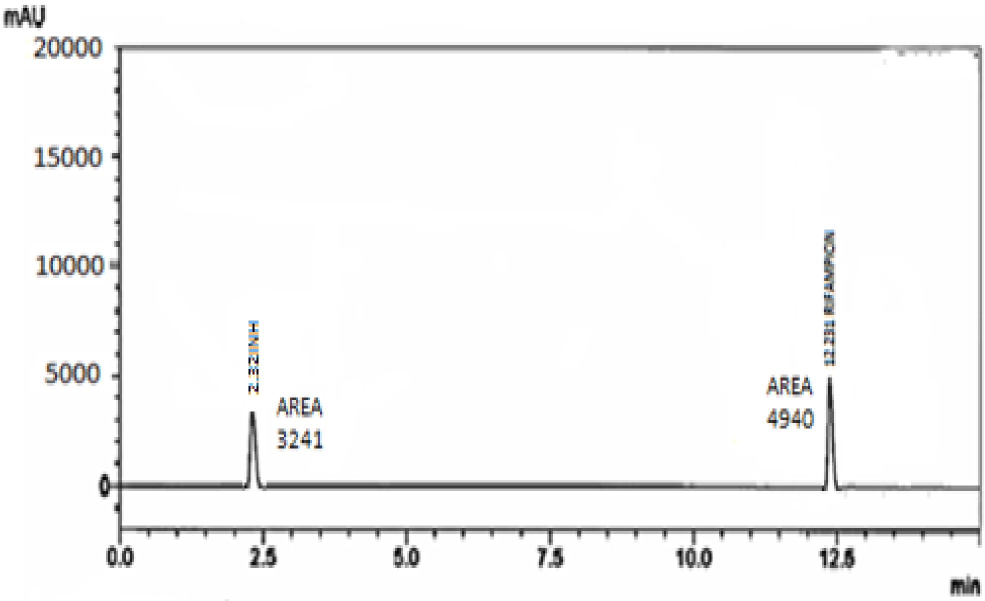


**Figure 61(b). 1$^{st}$hr**

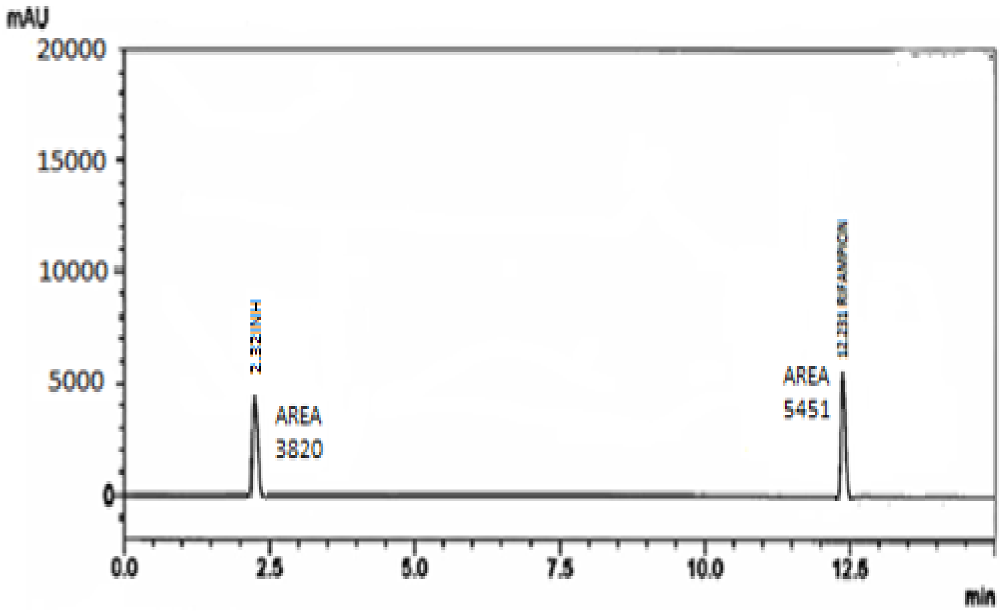


**Figure 61(c). 2nd hr**

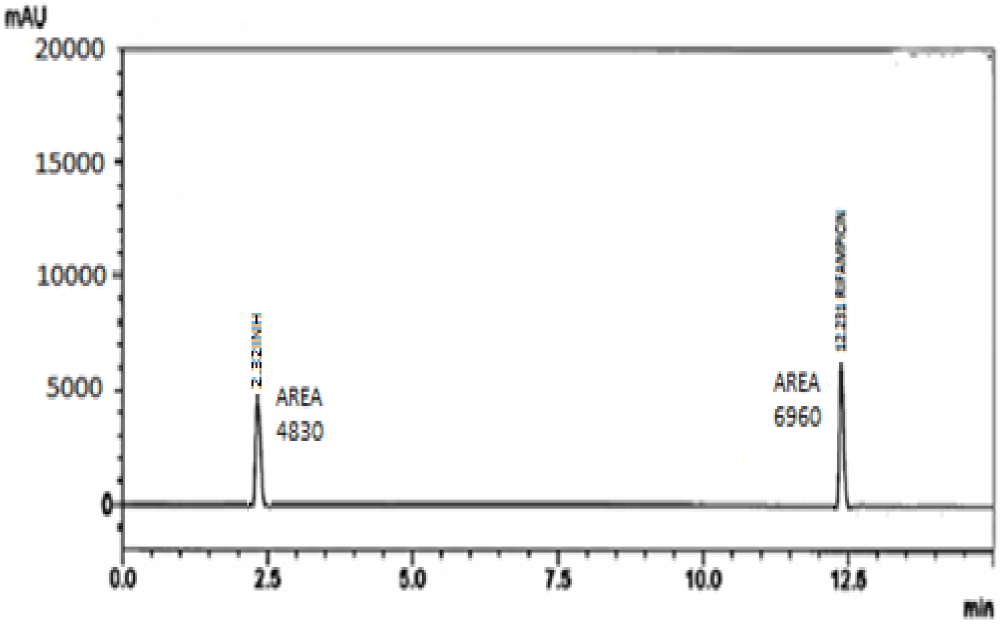


**Figure 61(d). 4th hr**

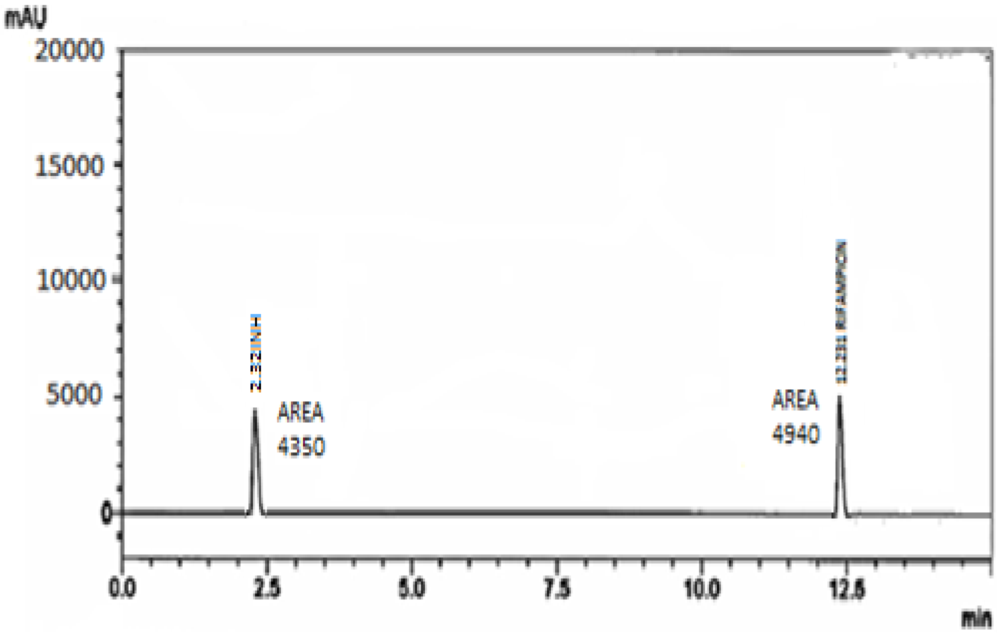


**Figure 61(e). 6th hr**

**Table 55 Group-III - Rifampicin - ascorbic acid (n=6, mean±SD)**

| Time(hr) | Concentration(μg/ml) | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| 0.5 | 6.0 | 6.2 | 6.4 | 6.1 | 6.5 | 6.2 | 6.2±0.115 |
| 1 | 8.1 | 8.6 | 8.4 | 8.5 | 8.4 | 8.2 | 8.3±0.155 |
| 2 | 8.7 | 8.1 | 8.3 | 8.5 | 8.3 | 8.7 | 8.0±0.1764 |
| 4 | 9.9 | 10.2 | 9.8 | 10.0 | 10.2 | 9.7 | 9.28±0.020 |
| 6 | 8.0 | 8.1 | 7.9 | 8.2 | 7.7 | 7.8 | 8.0±0.064 |
| 9 | 4.1 | 4.3 | 3.9 | 3.8 | 4.0 | 3.9 | 4.1±0.1155 |
| 12 | 2.7 | 1.9 | 2.3 | 2.5 | 2.6 | 2.0 | 2.3±0.2309 |

## HPLC Chromatograms

### Group-III - Rifampicin - ascorbic acid (n=6, mean±SD)

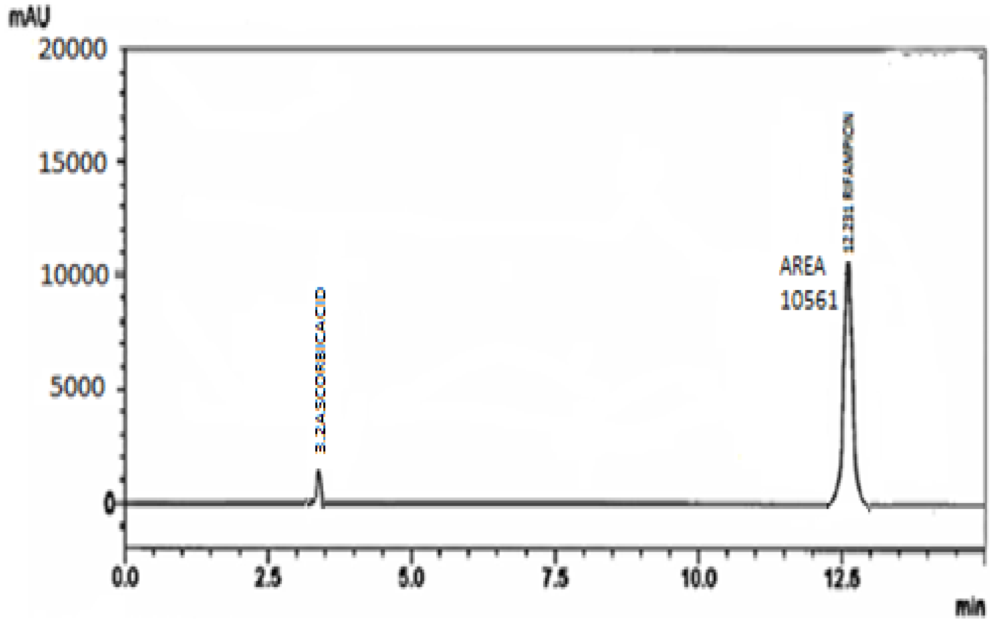


**Figure 62(a). 0.5hr**

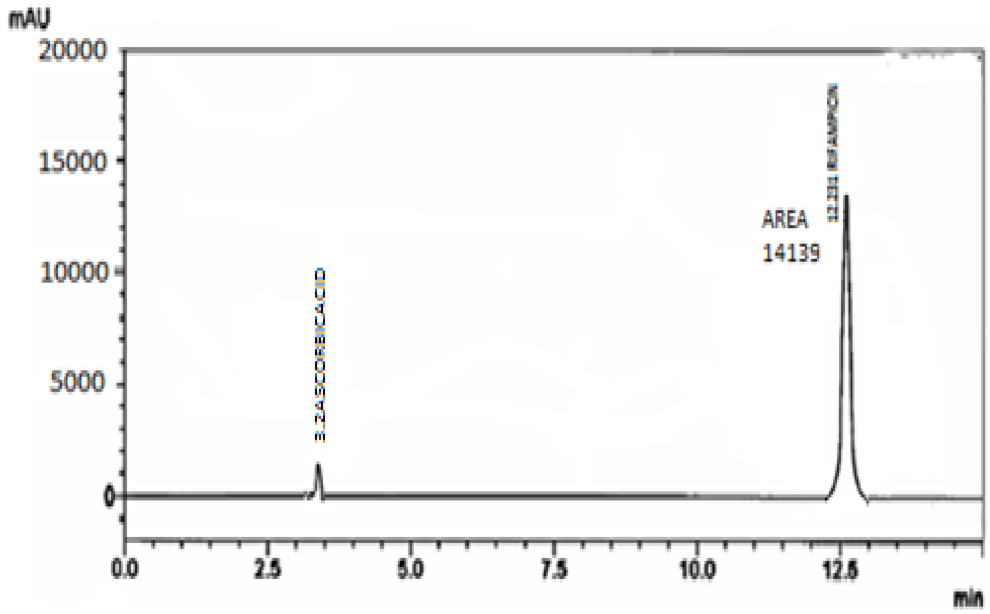


**Figure 62(b). 1$^{st}$ hr**

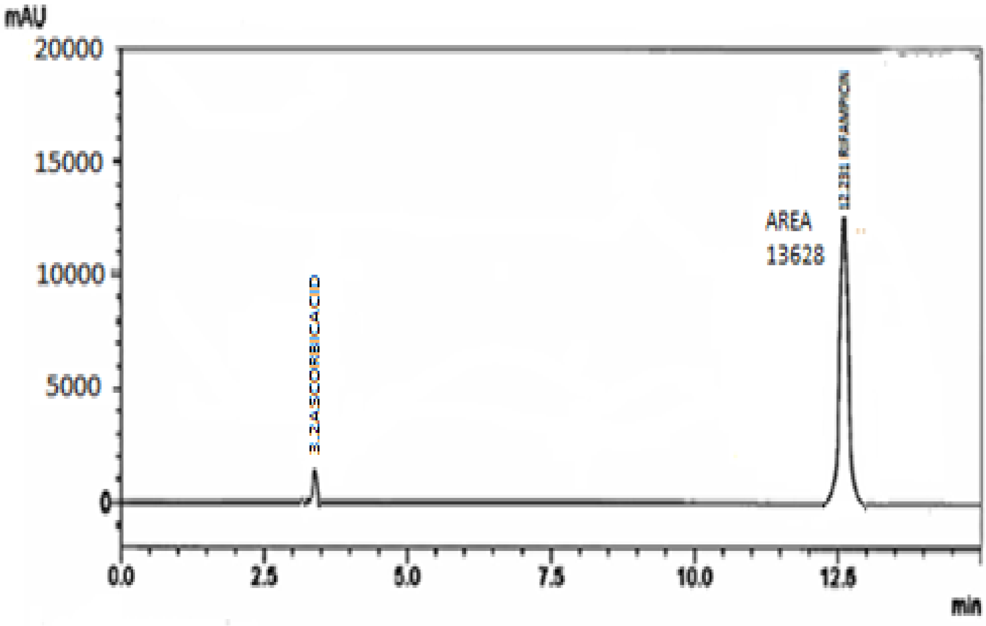


**Figure 62(c). $2^{nd}$hr**

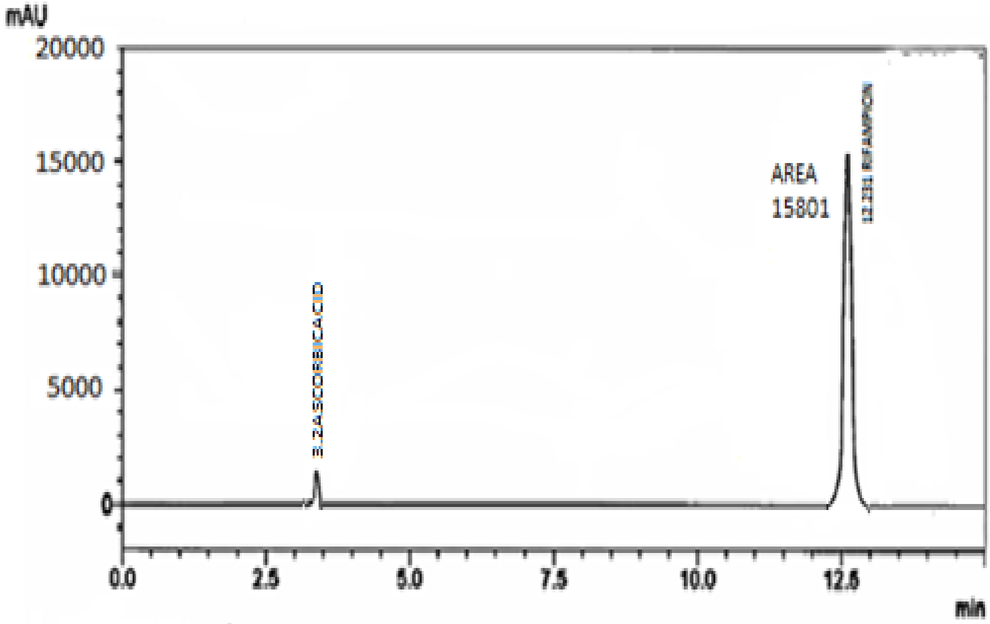


**Figure 62(d). $4^{th}$ hr**

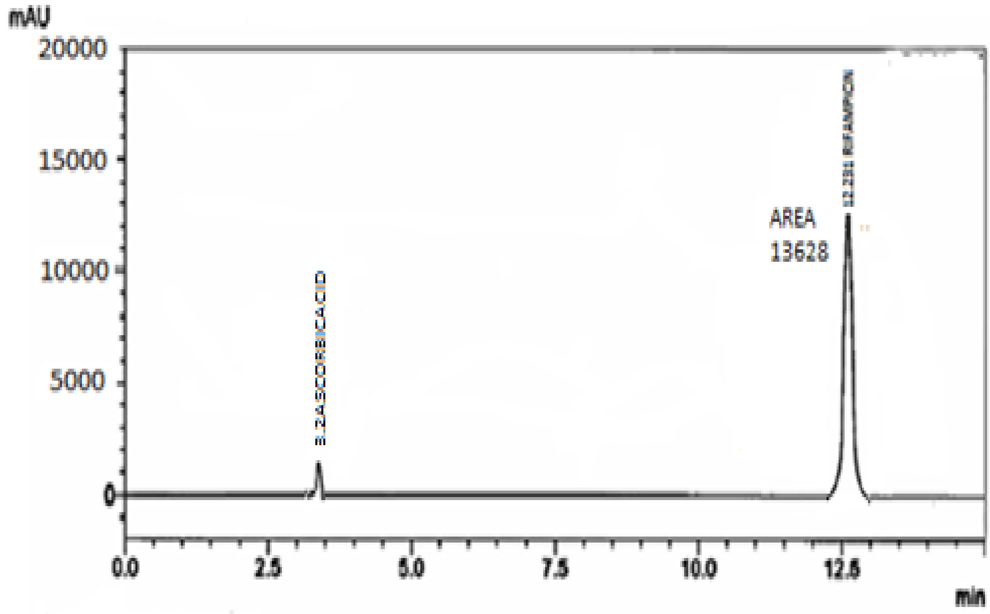


**Figure 62(e). 6th hr**

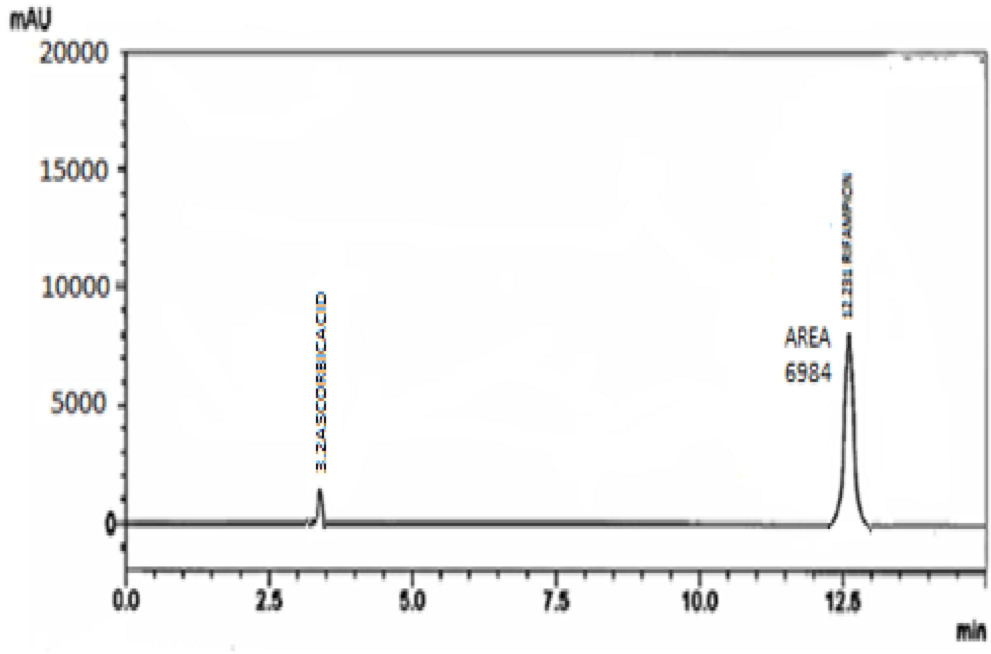


**Figure 62(f). 9thhr**

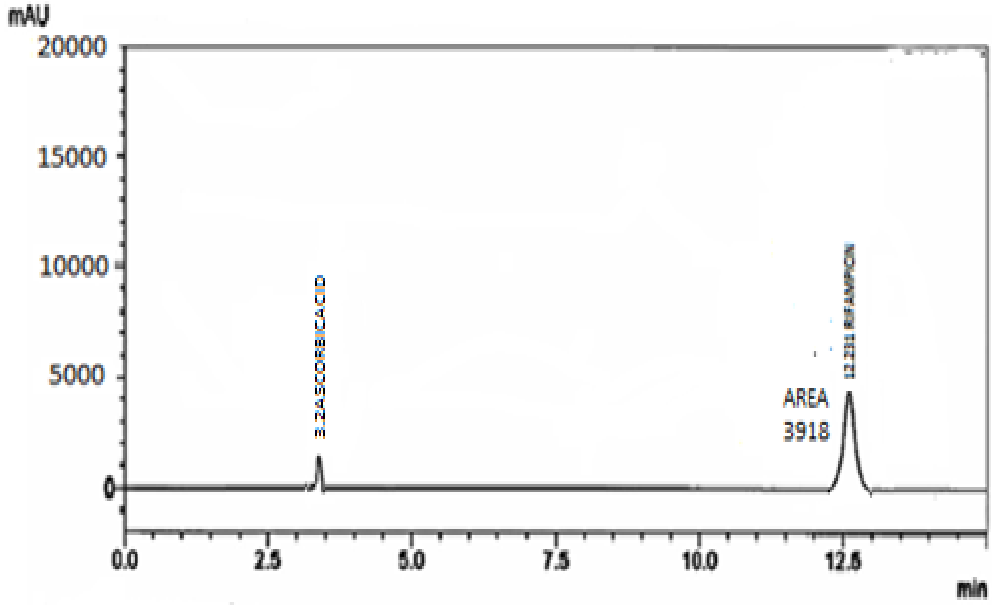


**Figure 62(g). 12th hr**

**Table 56 Group-IV -Rifampicin - ascorbic acid + isoniazid (n=6, mean±SD)**

| Time(hr) | Concentration(μg/ml) | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| 0.5 | 5.4 | 5.8 | 6.0 | 5.2 | 6.1 | 5.9 | 5.7±0.18 |
| 1 | 6.9 | 7.1 | 7.4 | 7.5 | 7.2 | 6.8 | 7.1±0.15 |
| 2 | 9.0 | 9.1 | 9.2 | 9.3 | 8.9 | 9.1 | 9.1±0.06 |
| 4 | 10.6 | 10.0 | 10.8 | 10.4 | 10.7 | 10.9 | 10.2±0.12 |
| 6 | 6.7 | 6.3 | 6.6 | 6.8 | 6.1 | 6.5 | 6.5±0.12 |
| 9 | 2.8 | 2.3 | 2.7 | 2.9 | 2.1 | 2.8 | 2.6±0.152 |
| 12 | 1.6 | 2.1 | 1.9 | 1.5 | 1.7 | 2.4 | 1.8±0.145 |

## HPLC Chromatograms

**Group-IV -Rifampicin - ascorbic acid + isoniazid (n=6, mean±SD)**

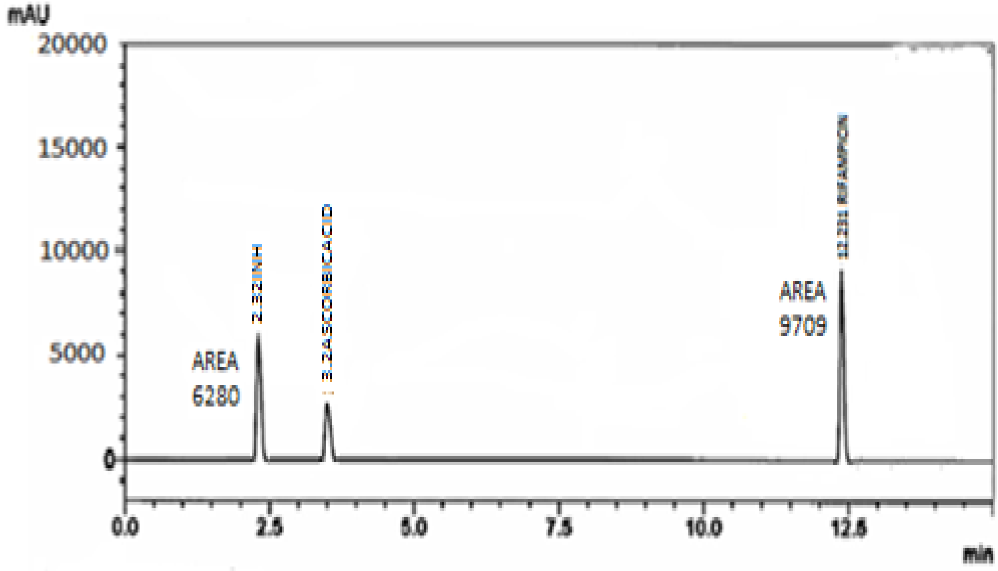


**Figure 63(a). 0.5hr**

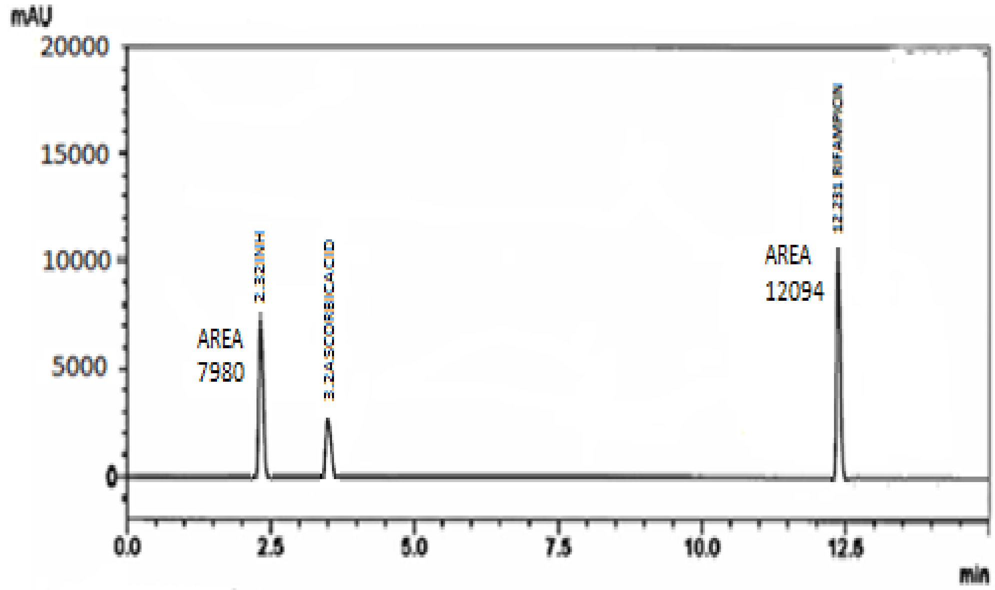


**Figure 63(b). 1st hr**

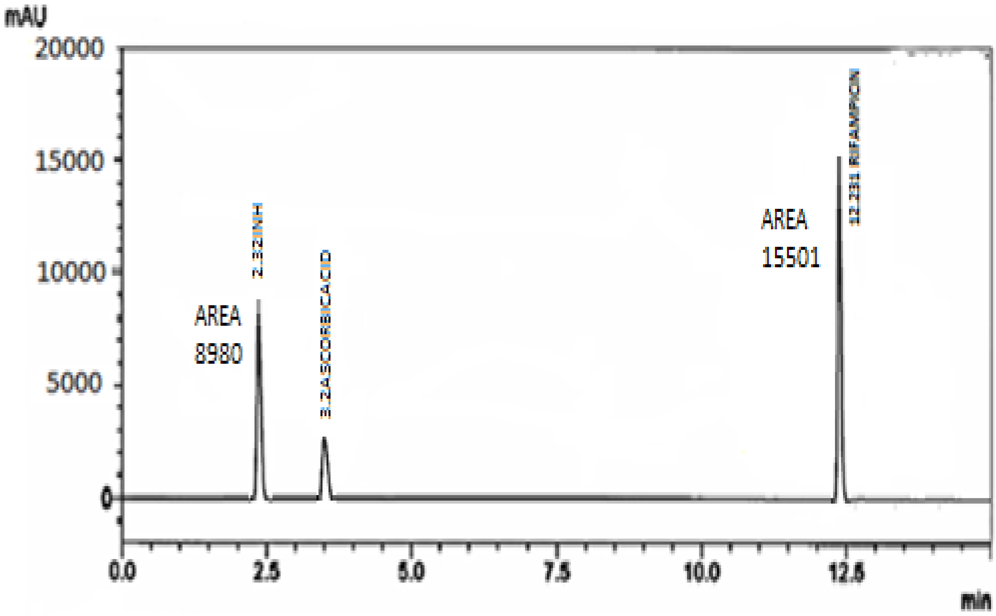


**Figure 63(c).  2nd hr**

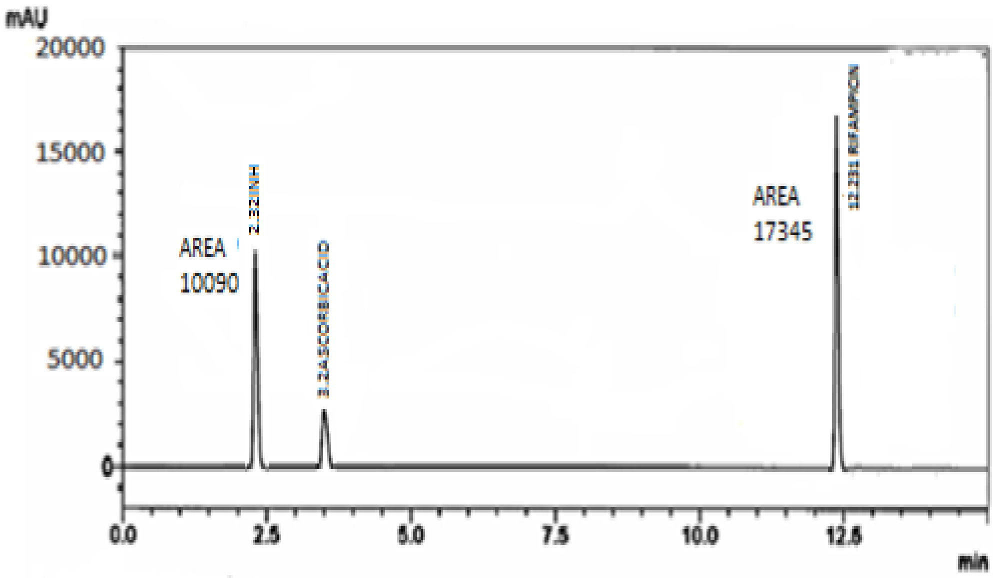


**Figure 63(d).  4th hr**

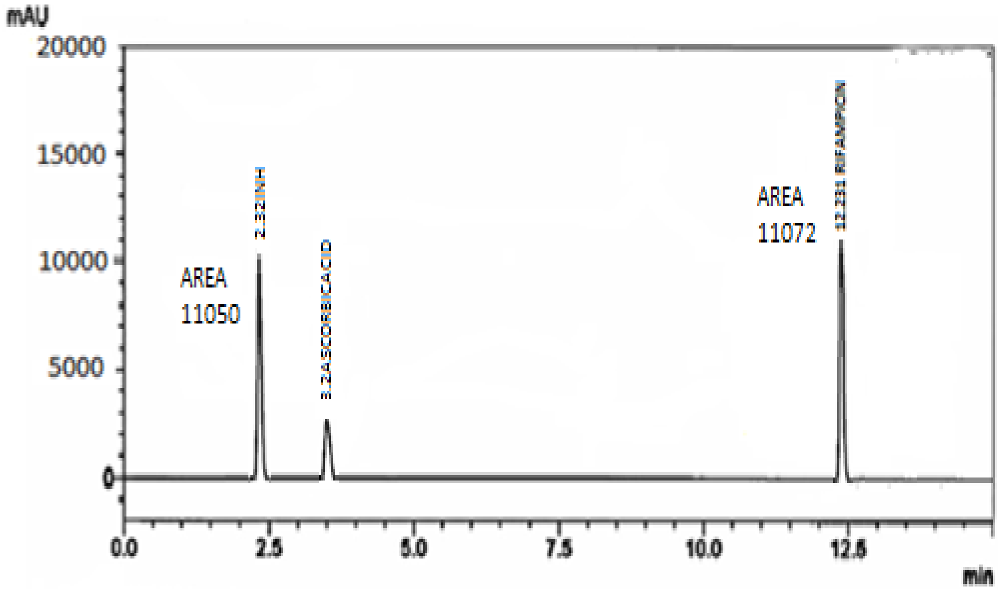


Figure 63(e). 6th hr

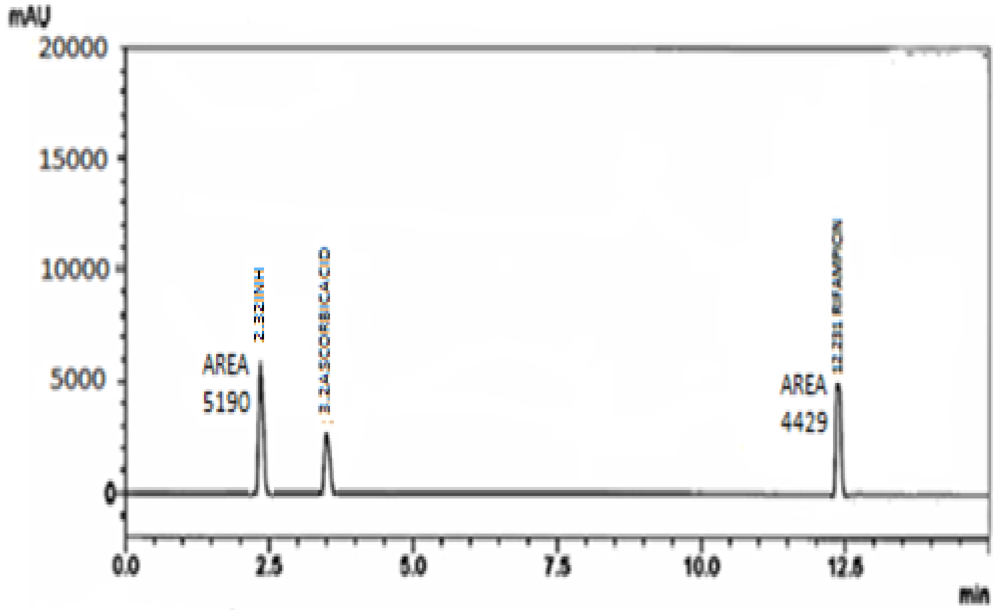


Figure 63(f). 9th hr

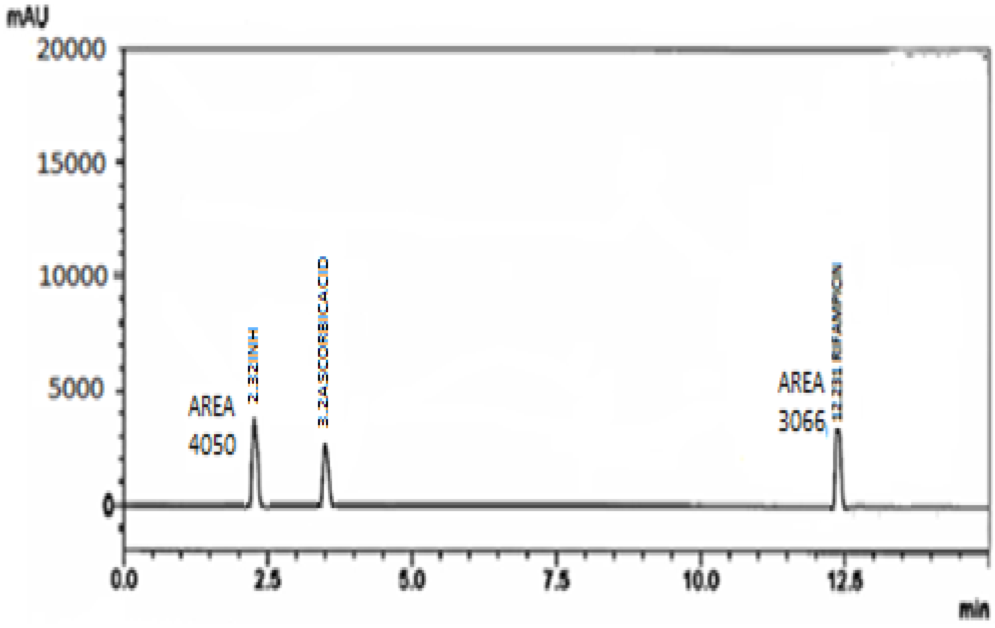


**Figure 63(g). 12$^{th}$ hr**

**Table 57 Group-V - Rifampicin nanoparticles (n=6, mean±SD)**

| Time(hr) | Concentration(µg/ml) | | | | | | Mean± S.D |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| 0.5 | 3.5 | 4.2 | 3.7 | 3.4 | 4.1 | 3.9 | 3.8±0.23 |
| 1 | 4.8 | 5.2 | 5.4 | 5.6 | 5.1 | 4.9 | 5.2±0.67 |
| 2 | 5.8 | 5.1 | 5.5 | 5.6 | 4.9 | 5.8 | 6.1±0.14 |
| 4 | 6.6 | 6.3 | 5.7 | 6.4 | 5.9 | 6.2 | 6.3±0.34 |
| 6 | 3.7 | 3.5 | 3.2 | 3.6 | 3.1 | 3.7 | 3.4±0.76 |
| 9 | 0.9 | 1.0 | 1.1 | 1.2 | 0.7 | 1.3 | 1.0±0. 57 |
| 12 | - | - | - | - | - | - | - |

**HPLC Chromatograms**

**Group-V - Rifampicin nanoparticles (n=6, mean±SD)**

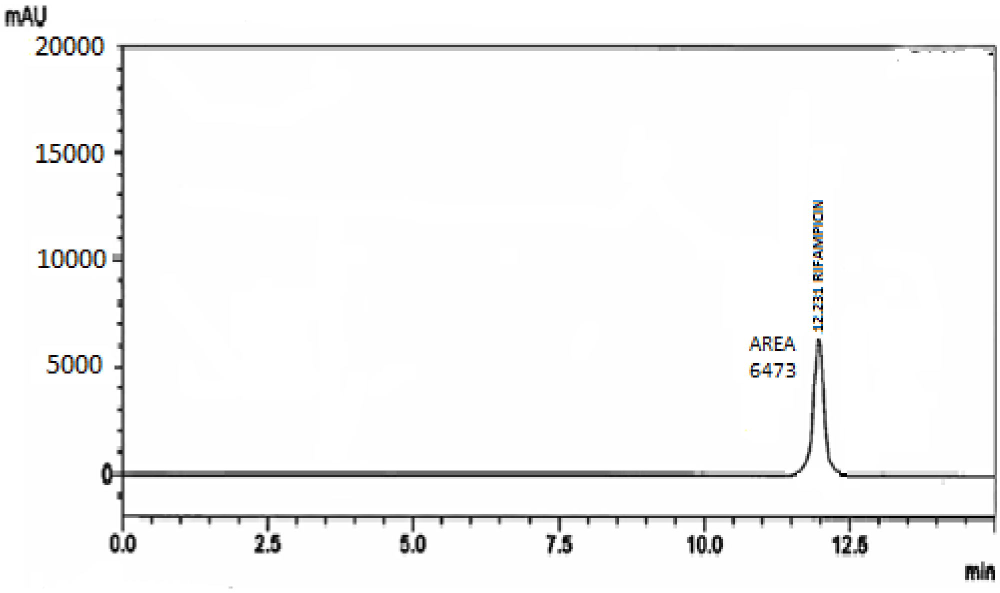


**Figure 64(a). 0.5hr**

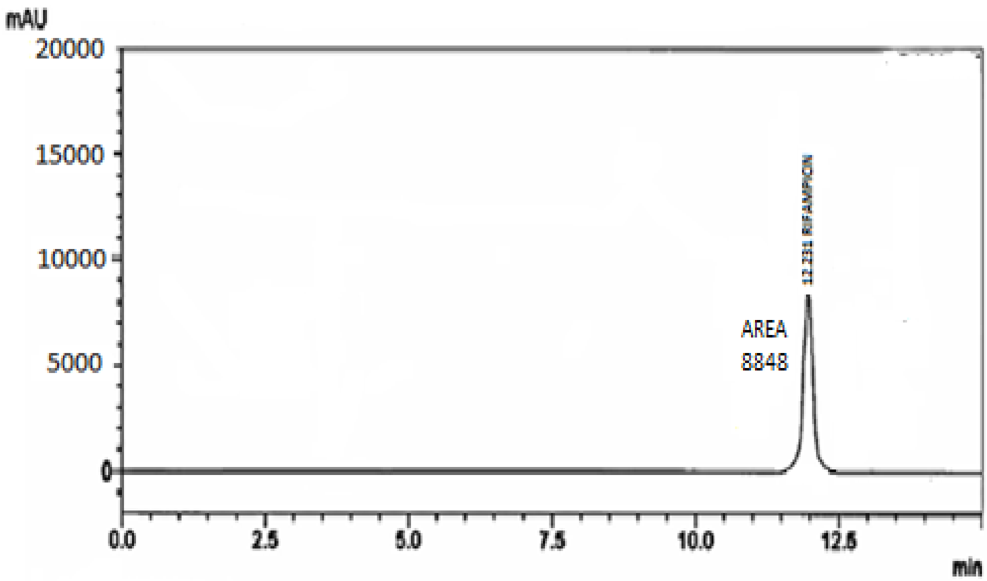


**Figure 64(b). 1st hr**

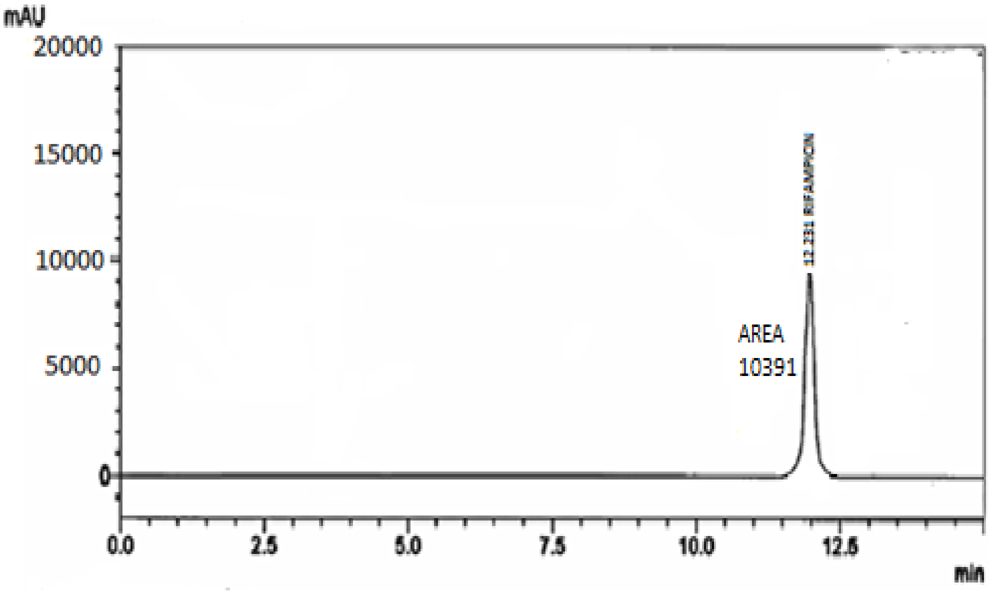


**Figure 64(c) . 2nd hr**

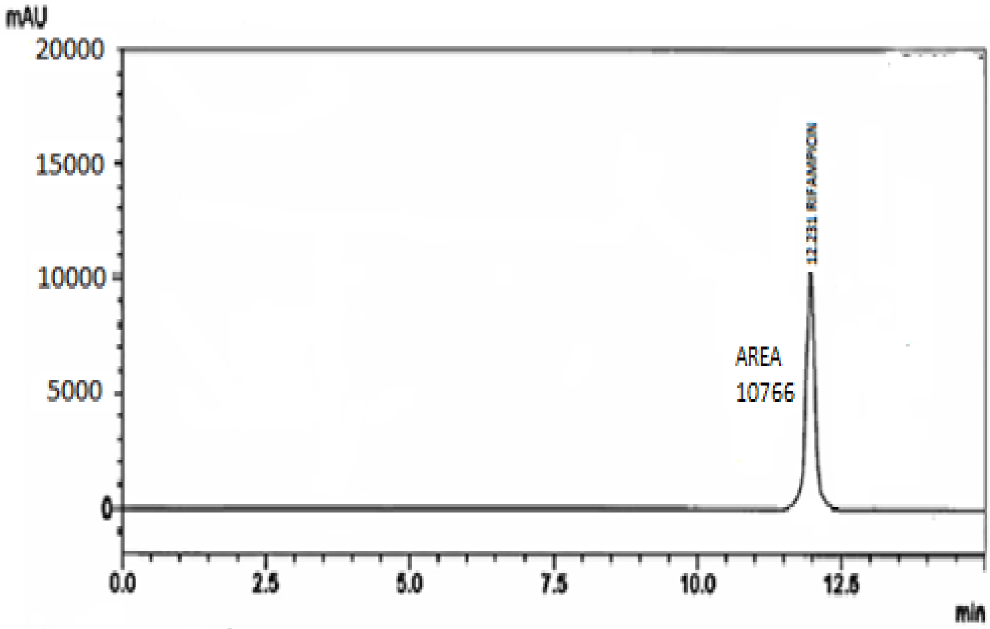


**Figure 64(d). 4th hr**

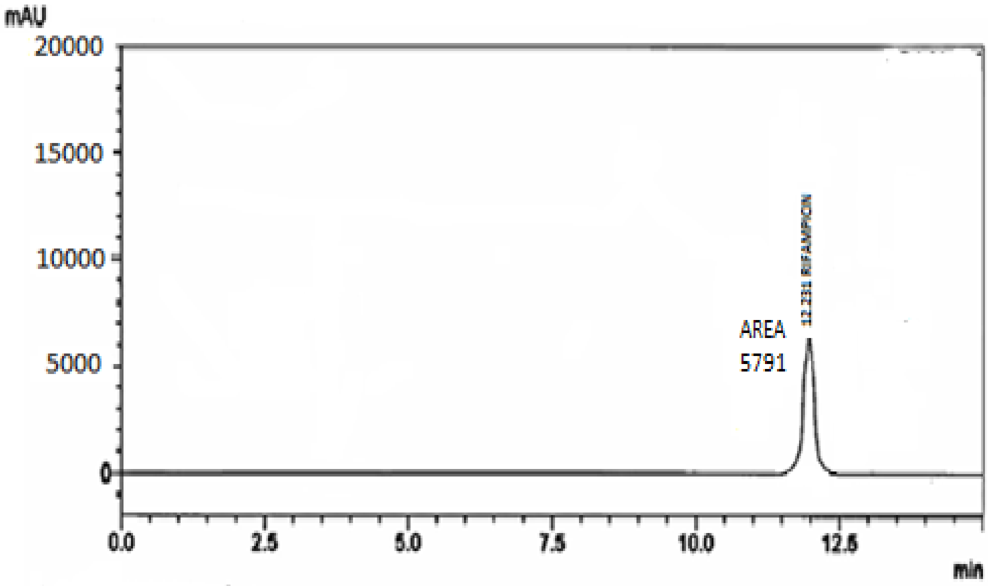


**Figure 64(e).  6th hr**

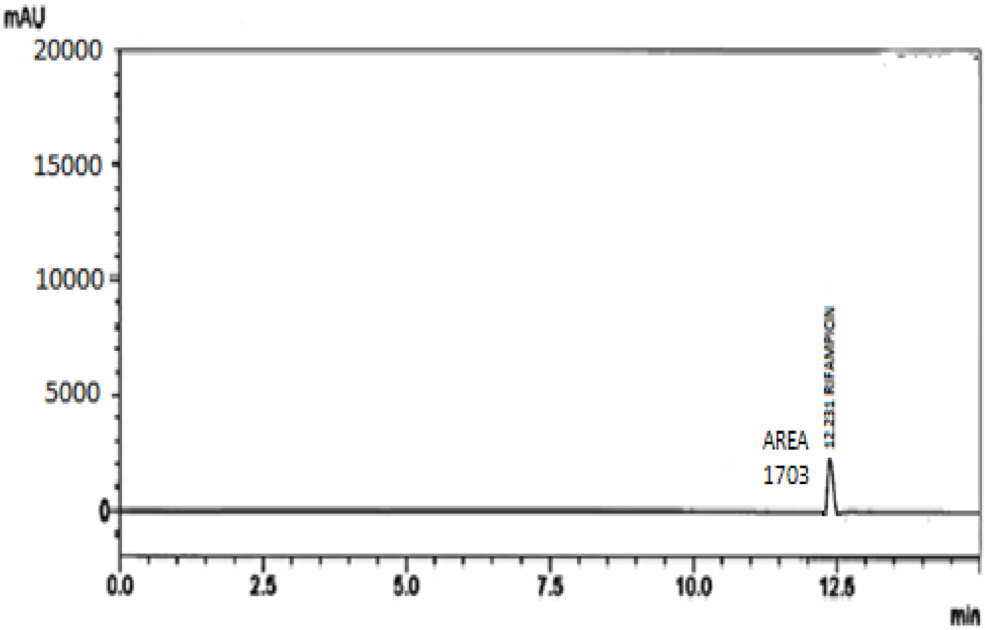


**Figure 64(f).  9th hr**

**Table 58 Group-VI- Rifampicin nanoparticles+ isoniazid (n=6, mean±SD)**

| **Time(hr)** | **Concentration(µg/ml)** | | | | | | **Mean± S.D** |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | |
| 0.5 | 2.7 | 2.3 | 2.6 | 2.8 | 2.5 | 2.4 | 2.7±0.87 |
| 1 | 4.0 | 4.2 | 4.4 | 3.9 | 4.5 | 4.1 | 4.2±0.56 |
| 2 | 5.1 | 5.3 | 4.9 | 4.9 | 4.9 | 4.9 | 5.1±0.46 |
| 4 | 3.9 | 4.0 | 4.1 | 3.7 | 3.8 | 4.4 | 5.6±0.54 |
| 6 | 2.9 | 3.0 | 2.8 | 2.7 | 2.9 | 3.1 | 2.9±0.85 |
| 9 | 1.9 | 1.2 | 2.2 | 1.8 | 1.0 | 2.5 | 2.0±0.23 |
| 12 | - | - | - | - | - | - | - |

**HPLC Chromatograms**

**Group-VI- Rifampicin nanoparticles+ isoniazid**

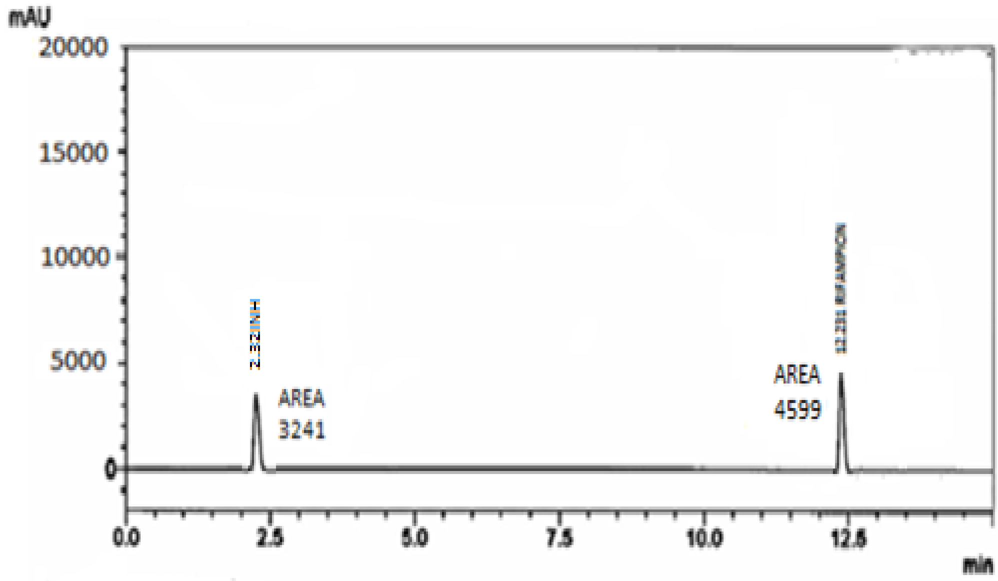


**Figure 65(a). 0.5hr**

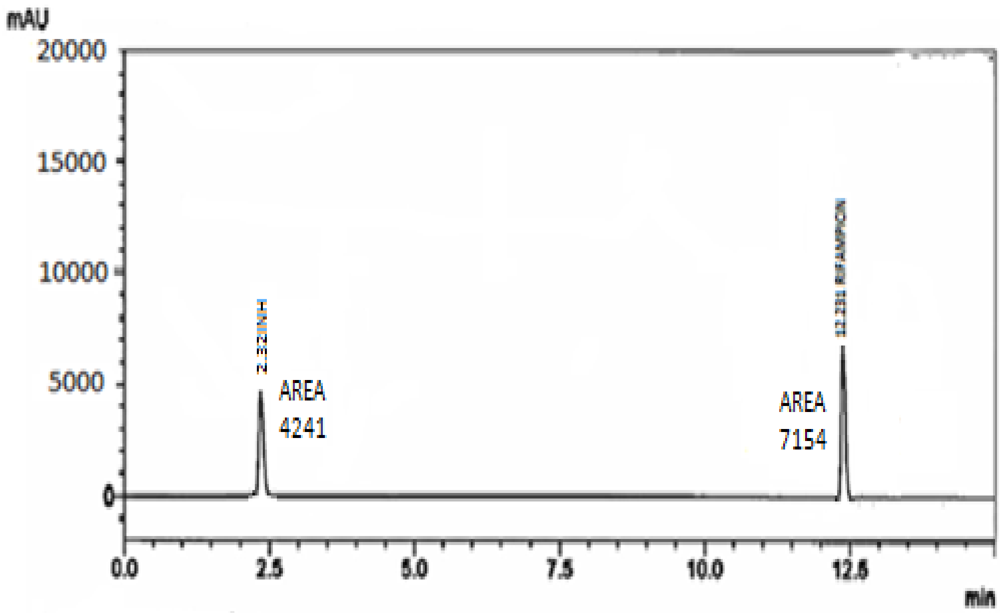


**Figure 65(b). 1st hr**

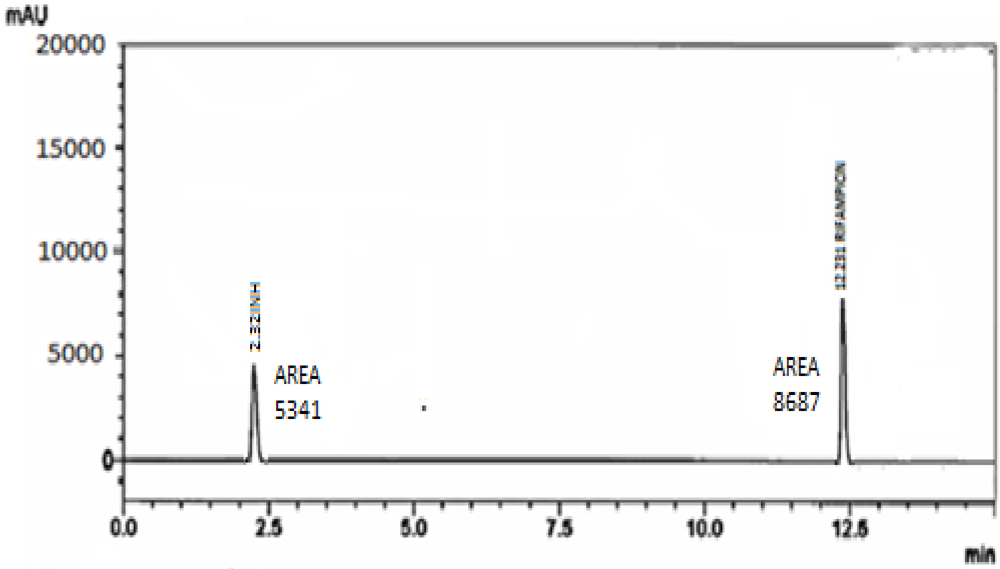


**Figure 65(c). 2nd hr**

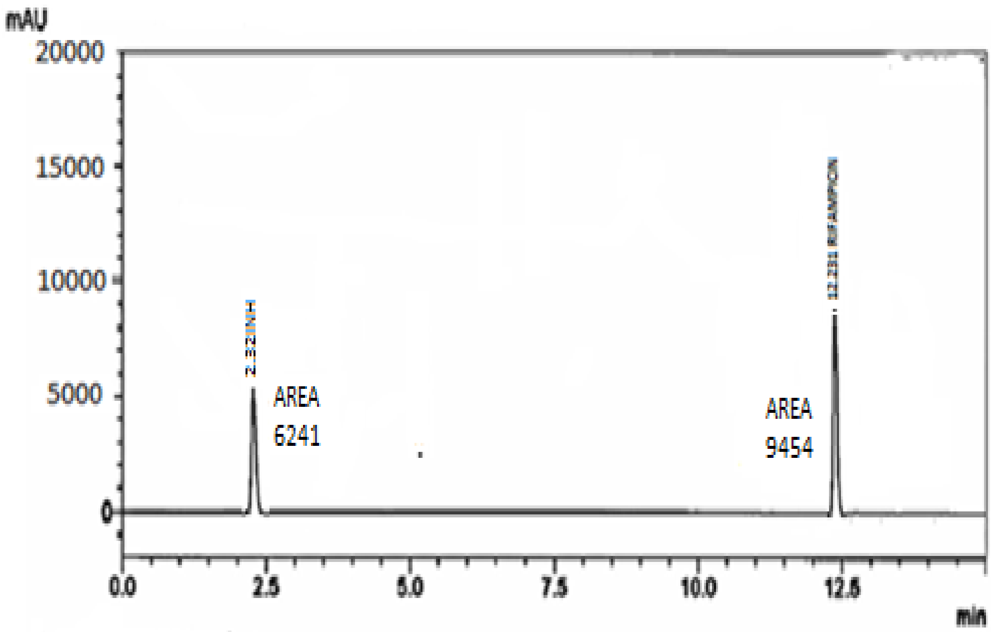


**Figure 65(d). 4th hr**

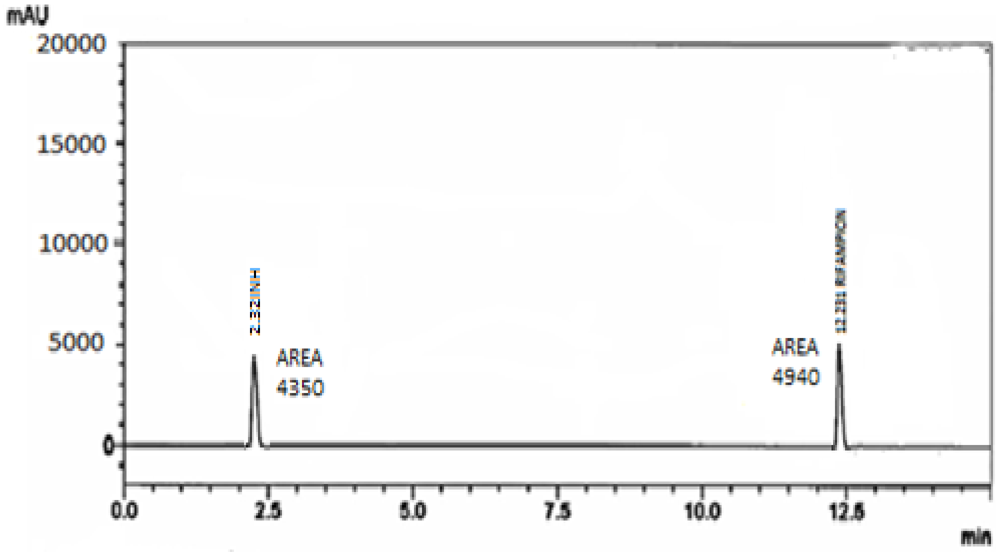


**Figure 65(e). 6th hr**

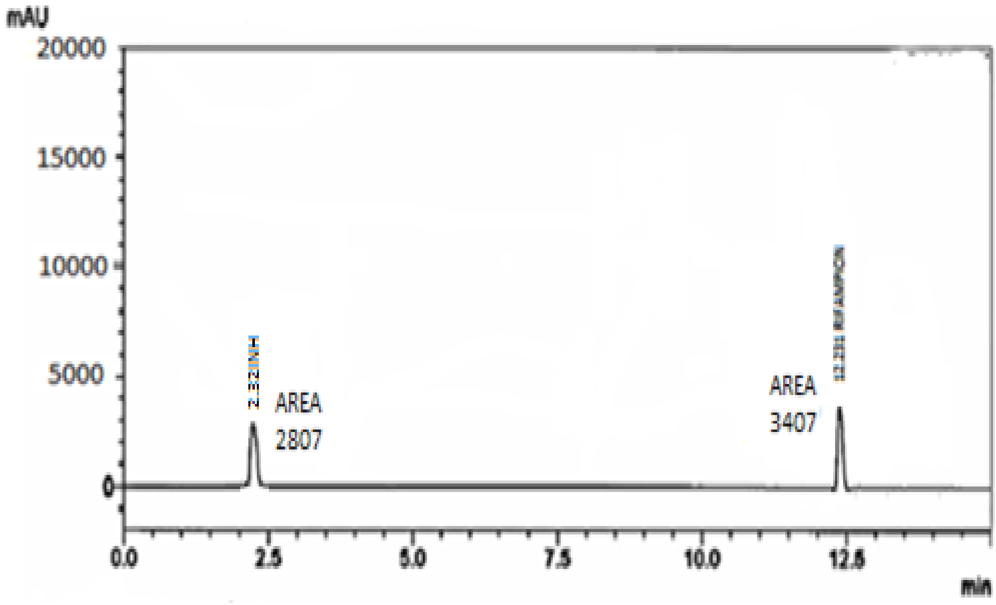


**Figure 65(f). 9th hr**

**Table 59 Group-VII –Rifampicin- ascorbic acid nanoparticles (n=6, mean±SD)**

| Time(hr) | Concentration(μg/ml) | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| 0.5 | 6.0 | 6.2 | 6.4 | 5.8 | 6.3 | 6.5 | 6.2±0.67 |
| 1 | 7.1 | 7.6 | 7.4 | 7.0 | 7.5 | 7.6 | 7.3±0.43 |
| 2 | 8.7 | 8.4 | 8.9 | 8.6 | 8.3 | 9.1 | 8.8±0.34 |
| 4 | 9.9 | 10.2 | 9.8 | 9.8 | 9.8 | 9.8 | 10.11±0.33 |
| 6 | 8.0 | 8.1 | 7.9 | 7.7 | 8.2 | 8.1 | 8.0±0.069 |
| 9 | 4.1 | 4.3 | 3.9 | 4.0 | 4.2 | 4.1 | 4.1±0. 15 |
| 12 | 2.7 | 1.9 | 2.3 | 2.5 | 2.0 | 2.4 | 2.3±0.1 9 |

**HPLC Chromatograms**

**Group-VII –Rifampicin- ascorbic acid nanoparticles**

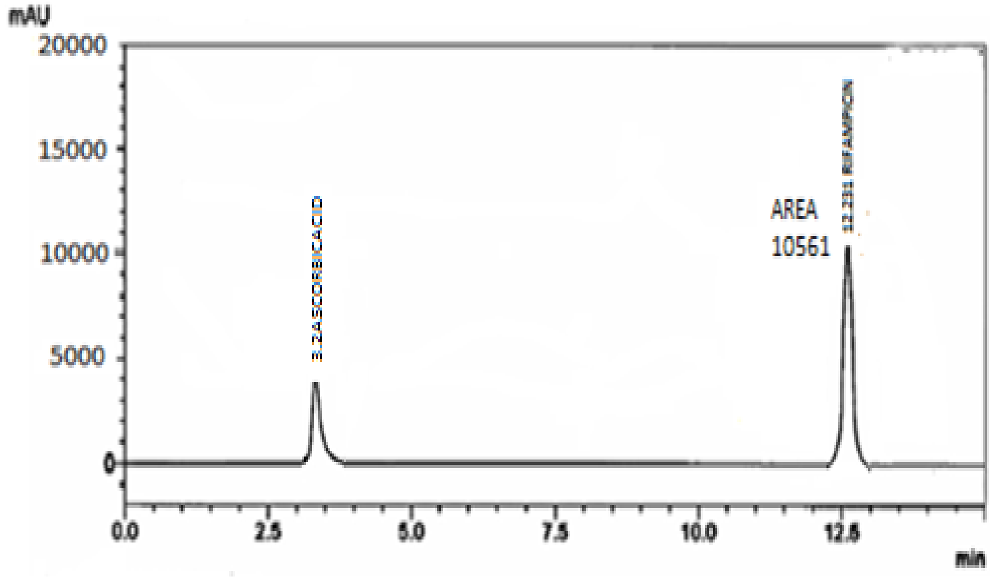


**Figure 66(a). 0.5 hr**

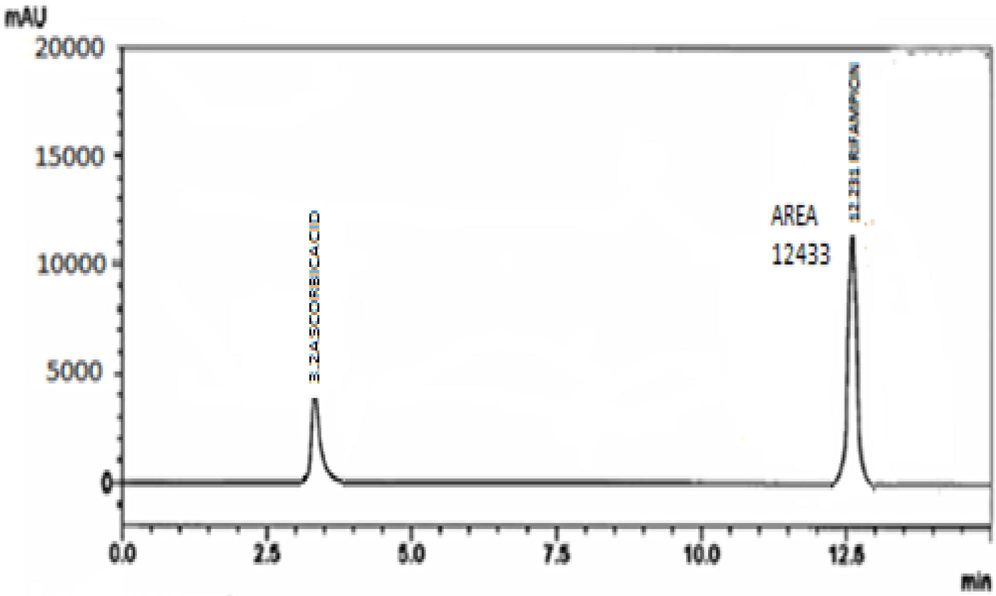


**Figure 66(b). 1$^{st}$ hr**

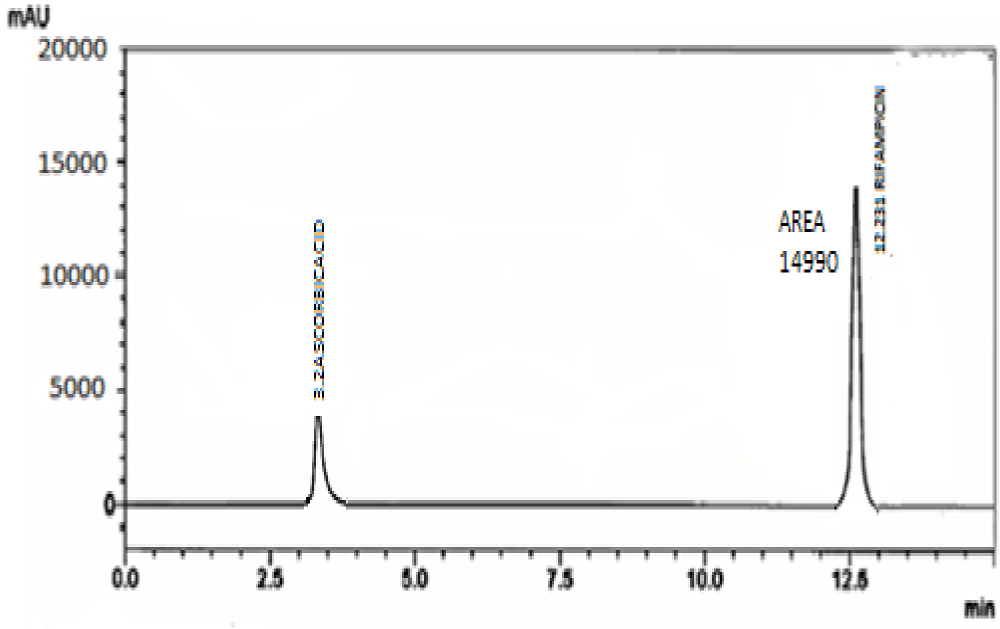


**Figure 66(c). 2nd hr**

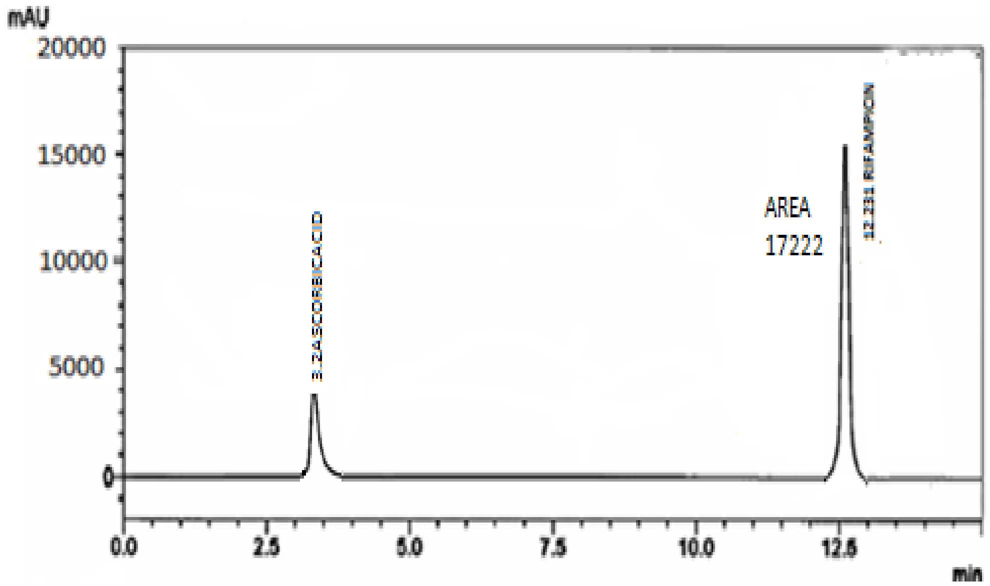


**Figure 66(d). 4th hr**

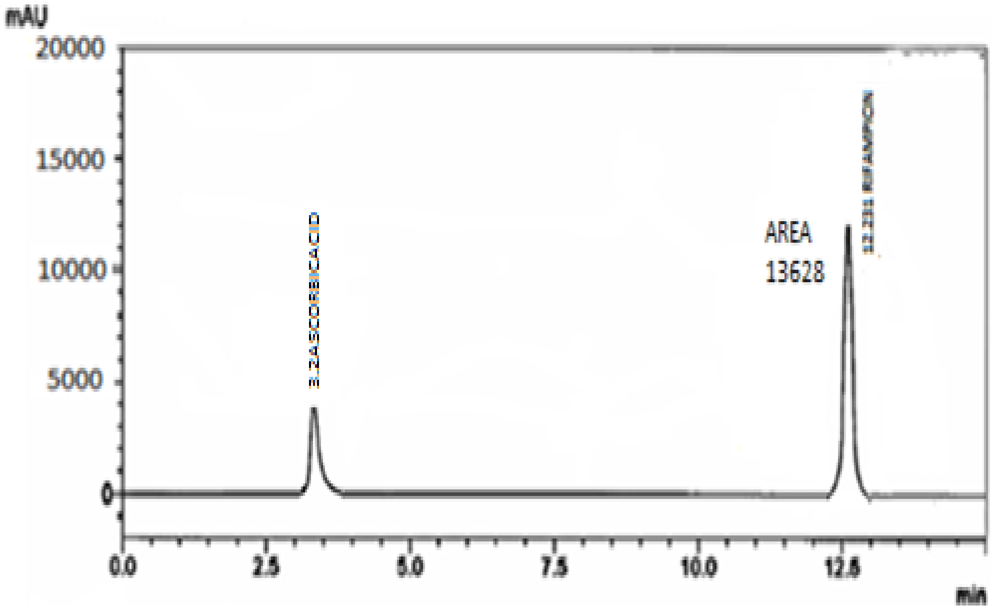


**Figure 66(e). 6th hr**

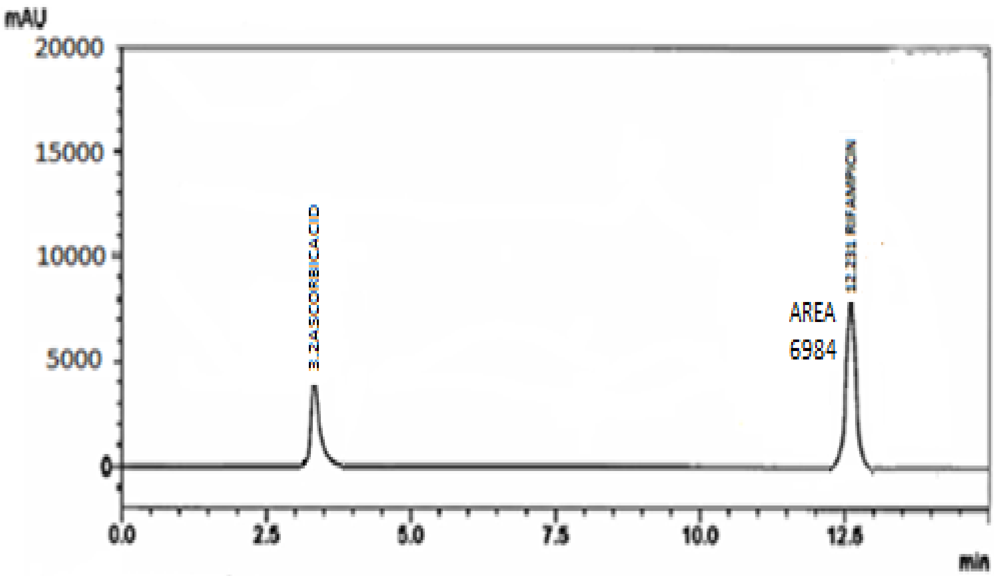


**Figure 66(f). 9th hr**

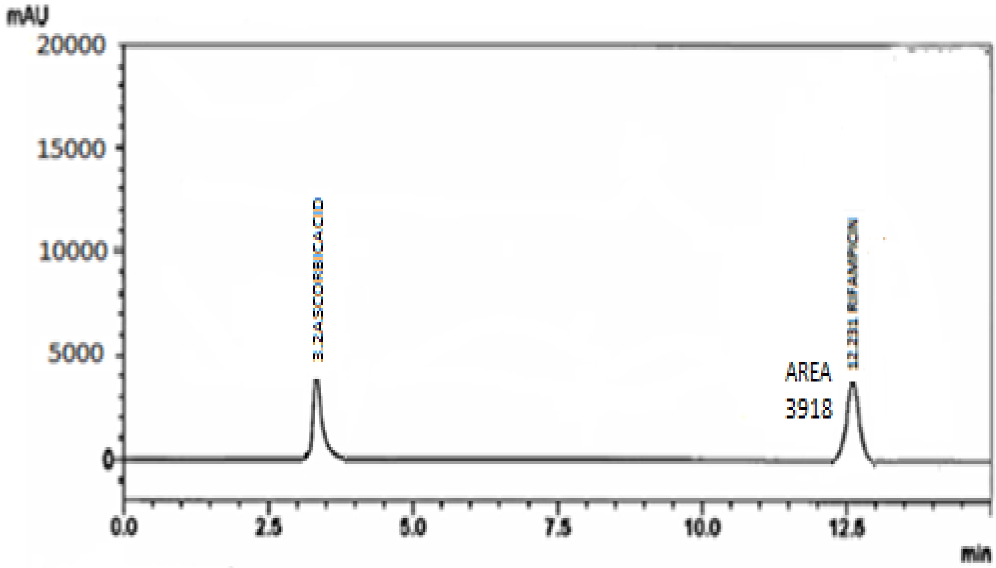


**Figure 66(g). 12th hr**

**Table 60 Group-VIII- Rifampicin-ascorbic acid nanoparticles + isoniazid**

**(n=6, mean±SD)**

| **Time(hr)** | **Concentration(µg/ml)** | | | | | | **Mean±S.D** |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | |
| 0.5 | 5.2 | 5.8 | 6.2 | 6.0 | 5.1 | 6.1 | 6.0±0.46 |
| 1 | 6.9 | 7.4 | 7.4 | 6.6 | 7.5 | 7.6 | 7.4±0.98 |
| 2 | 9.0 | 9.1 | 9.2 | 9.2 | 9.2 | 9.2 | 9.1±0.56 |
| 4 | 8.6 | 8.0 | 8.8 | 8.4 | 8.1 | 8.9 | 10.4±0.34 |
| 6 | 6.9 | 6.3 | 6.6 | 6.7 | 6.2 | 6.9 | 6.7±0.56 |
| 9 | 3.8 | 2.3 | 3.7 | 3.5 | 2.4 | 3.9 | 3.8±0.45 |
| 12 | 1.6 | 2.1 | 2.1 | 1.4 | 2.0 | 2.4 | 2.1±0.34 |

## HPLC Chromatograms

### Group-VIII- Rifampicin-ascorbic acid nanoparticles + isoniazid

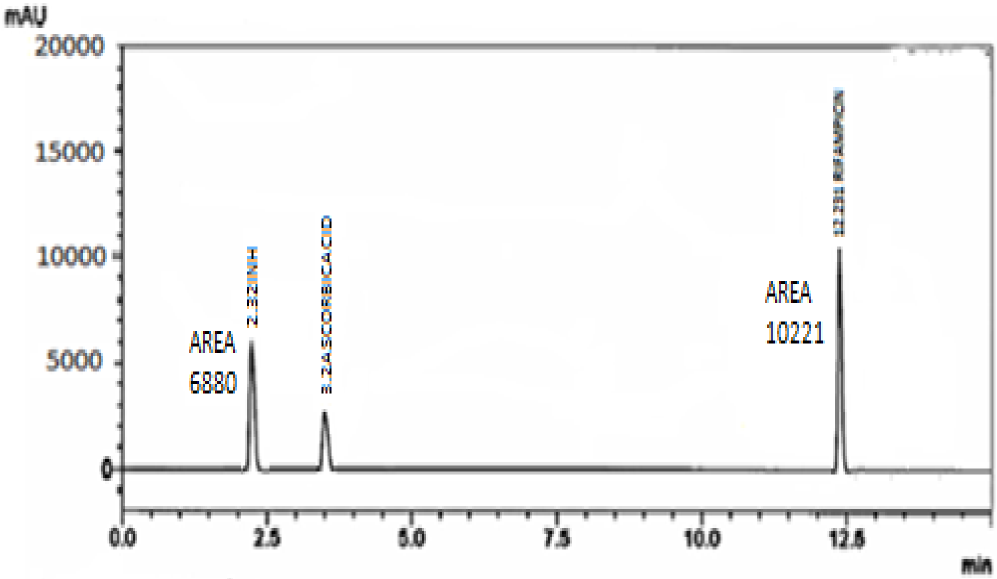


**Figure 67(a). 0.5hr**

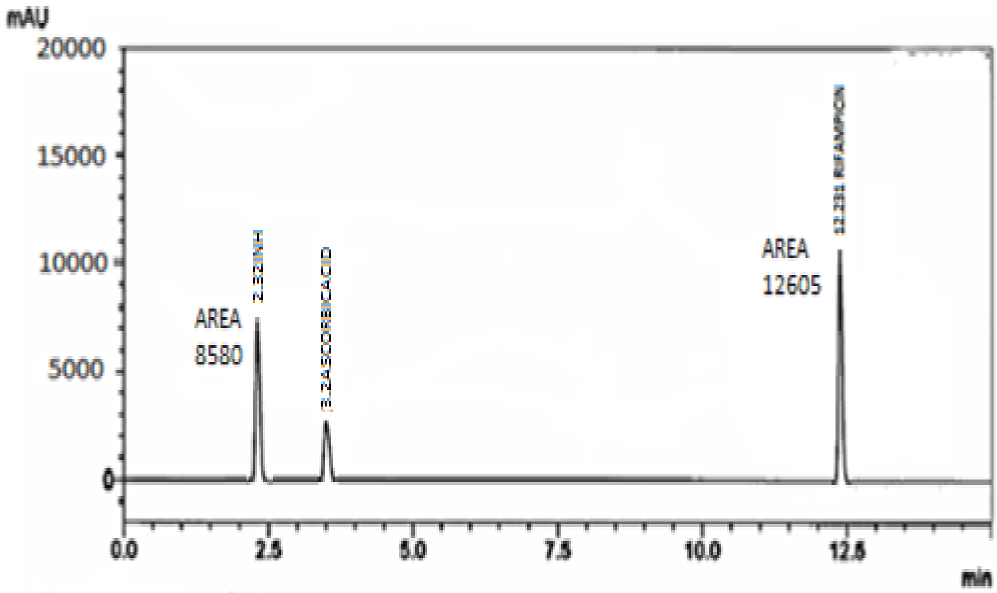


**Figure 67(b). 1$^{st}$ hr**

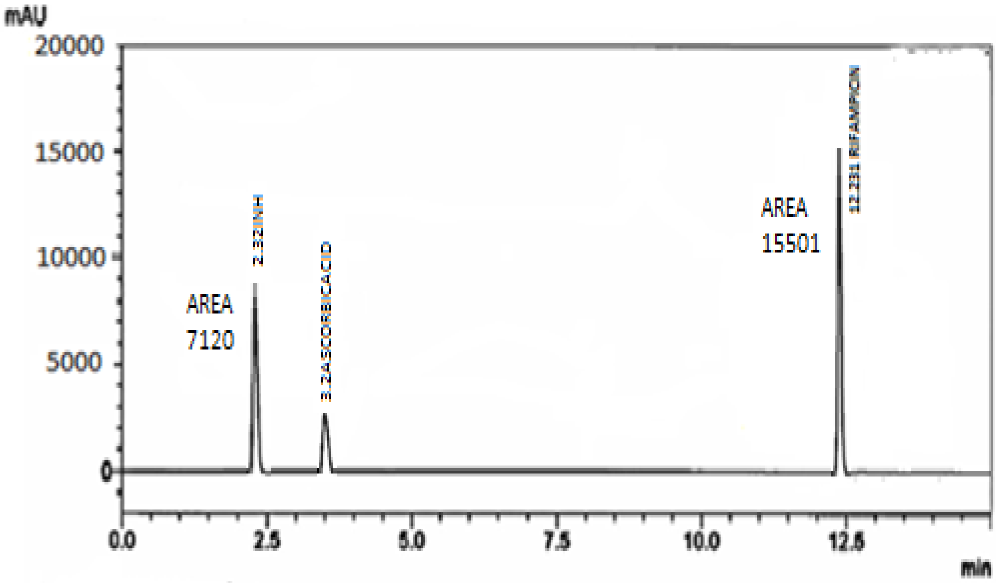


**Figure 67(c). 2nd hr**

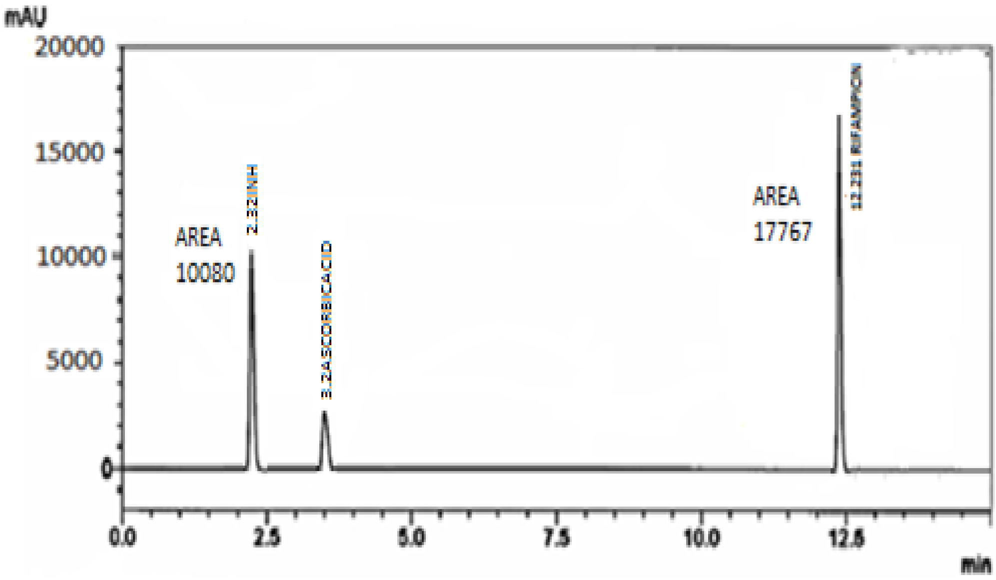


**Figure 67(d). 4th hr**

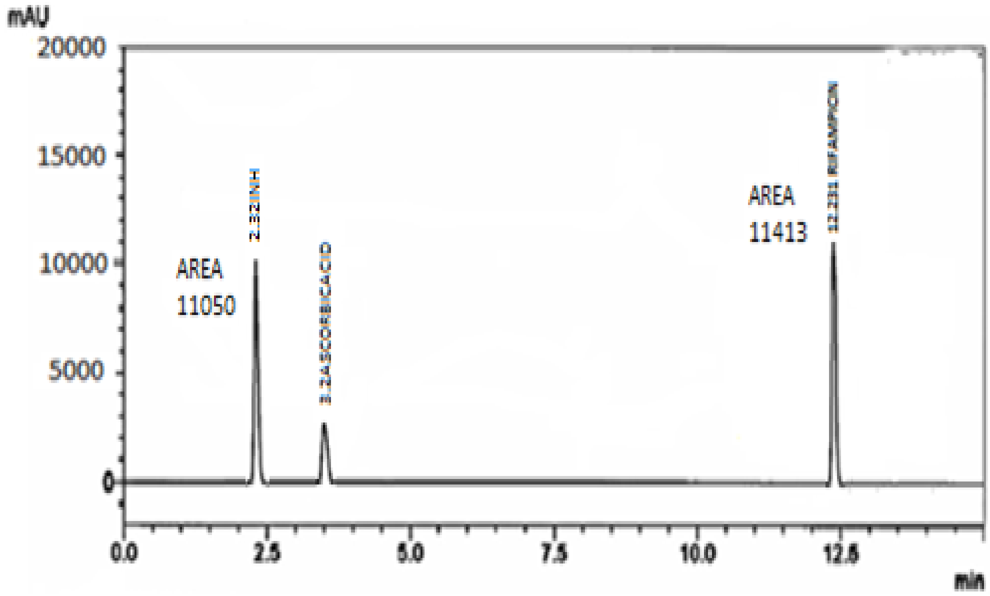


**Figure 67(e). 6$^{th}$ hr**

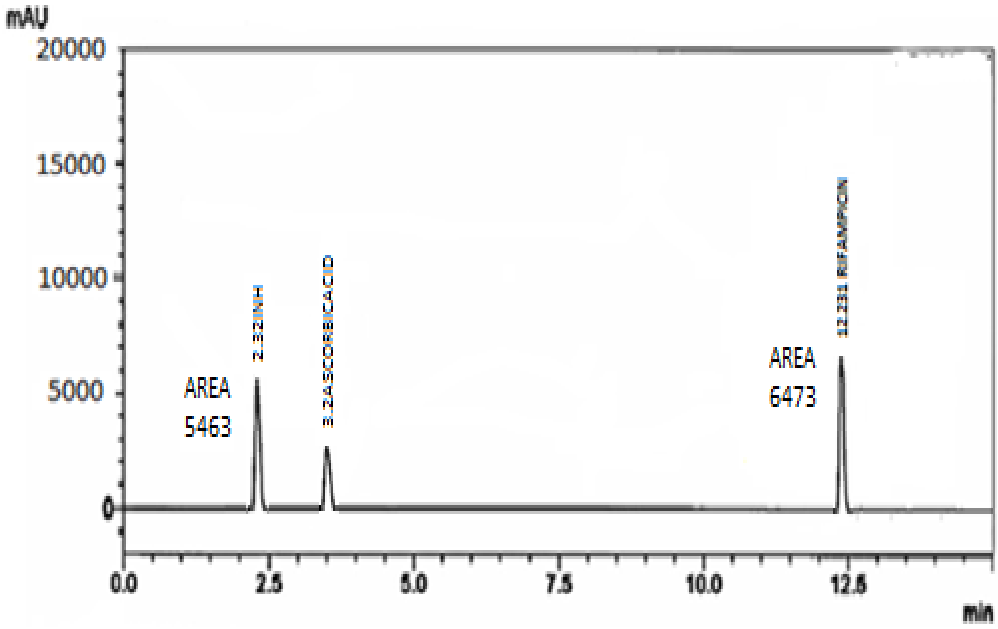


**Figure 67(f). 9$^{th}$ hr**

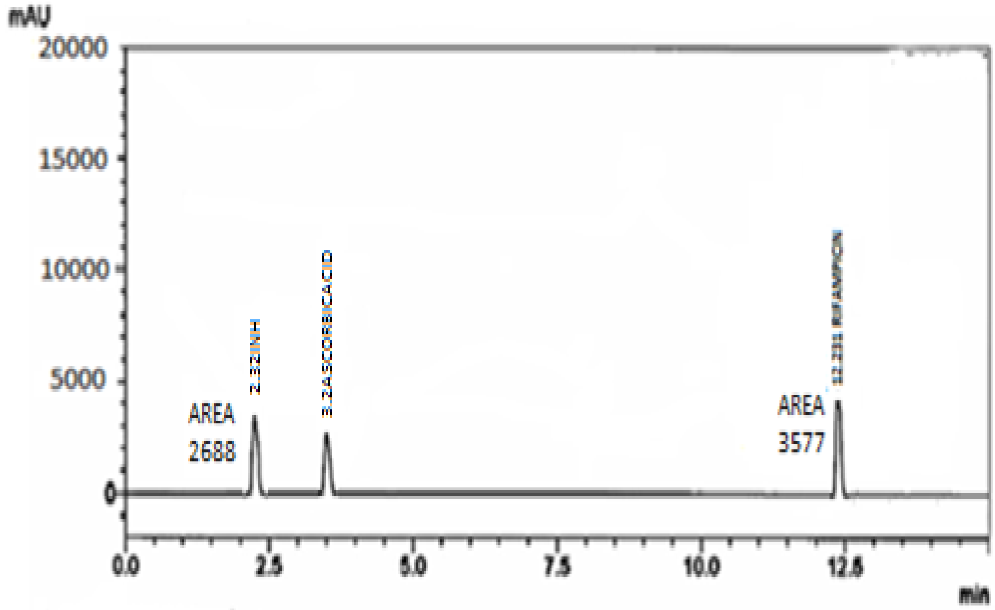


**Figure 67(g). 12th hr**

**Data of pharmacokinetic study**

**Table 61 Group I - Rifampicin alone (n=6, mean±SD)**

| **S.No** | **Parameters** | **Trails** | | | | | | **Mean±S.D** |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **6** | |
| 1 | $K_e$(1/h) | 0.398 | 0.335 | 0.358 | 0.397 | 0.334 | 0.360 | 0.363±0.0184 |
| 2 | $K_a$ | 0.419 | 0.425 | 0.431 | 0.418 | 0.424 | 0.433 | 0.425±0.0034 |
| 3 | $t_{1/2\ (h)}$ | 1.70 | 1.85 | 1.92 | 1.60 | 1.84 | 1.94 | 1.823±0.064 |
| 4 | $V_{d\ (l)}$ | 0.91 | 0.93 | 0.95 | 0.90 | 0.92 | 0.97 | 0.93±0.011 |
| 5 | $T_{max\ (h)}$ | 1.20 | 1.30 | 1.50 | 1.10 | 1.2 | 1.7 | 1.333±0.088 |
| 6 | $C_{max}$(µg/ml) | 5.90 | 5.82 | 6.0 | 5.89 | 5.81 | 6.2 | 5.906±0.052 |
| 7 | $AUC_{0-12}$(µg/mlh) | 34.31 | 36.21 | 31.36 | 34.30 | 36.20 | 31.38 | 33.96±1.411 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 60.61 | 62.60 | 59.94 | 60.60 | 62.59 | 59.96 | 61.05±0.798 |

**Table 62 Group II - Rifampicin+ isoniazid ((n=6, mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **6** | |
| 1 | $K_e$(1/h) | 0.295 | 0 .299 | 0.310 | 0.294 | 0.298 | 0.312 | 0.301±0.004 |
| 2 | $K_a$ | 0.315 | 0.318 | 0.321 | 0.314 | 0.317 | 0.323 | 0.318±0.001 |
| 3 | $t_{1/2(h)}$ | 2.7 | 2.4 | 2.5 | 2.6 | 2.3 | 2.7 | 2.533±0.088 |
| 4 | $V_{d\ (l)}$ | 0.83 | 0.87 | 0.89 | 0.82 | 0.86 | 0.91 | 0.863±0.017 |
| 5 | $T_{max\ (h)}$ | 1.61 | 1.63 | 1.68 | 1.60 | 1.62 | 1.70 | 1.64±0.020 |
| 6 | $C_{max}$(µg/ml) | 4.10 | 4.16 | 4.0 | 4.09 | 4.15 | 4.2 | 4.086±0.046 |
| 7 | $AUC_{0-12}$(µg/mlh) | 29.13 | 28.16 | 26.15 | 29.12 | 28.15 | 26.17 | 27.81±0.877 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 50.14 | 54.31 | 56.14 | 50.13 | 54.30 | 56.16 | 53.53±1.775 |

**Table 63 Group III- Rifampicin+ ascorbic acid (n=6, mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| 1 | $K_e$(1/h) | 0.418 | 0.421 | 0.433 | 0.417 | 0.420 | 0.435 | 0.424±0.004 |
| 2 | $K_a$ | 0.525 | 0.533 | 0.545 | 0.524 | 0.532 | 0.547 | 0.534±0.005 |
| 3 | $t_{1/2(h)}$ | 0.99 | 0.97 | 1.0 | 0.98 | 0.96 | 1.2 | 1.594±0.008 |
| 4 | $V_{d\ (l)}$ | 0.99 | 1.0 | 1.2 | 0.98 | 0.9 | 1.4 | 1.603±0.068 |
| 5 | $T_{max\ (h)}$ | 1.0 | 1.1 | 1.3 | 0.9 | 1.0 | 1.5 | 1.133±0.088 |
| 6 | $C_{max}$(µg/ml) | 9.24 | 9.28 | 9.31 | 9.23 | 9.27 | 9.33 | 9.276±0.020 |
| 7 | $AUC_{0-12}$(µg/mlh) | 64.68 | 68.16 | 74.6 | 64.67 | 68.15 | 74.8 | 69.146±2.906 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 78.90 | 81.77 | 86.7 | 78.89 | 81.76 | 86.9 | 82.48±2.099 |

**Table 64 Group IV-Rifampicin-ascorbic acid+ Isoniazid (n=6, mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **6** | |
| 1 | $K_e$(1/h) | 0.615 | 0.614 | 0.616 | 0.614 | 0.613 | 0.618 | 0.518±.006 |
| 2 | $K_a$ | 0.514 | 0.511 | 0.531 | 0.513 | 0.510 | 0.533 | 0.615±0.0005 |
| 3 | $t_{1/2(h)}$ | 0.95 | 0.97 | 0.94 | 0.92 | 0.93 | 1.01 | 0.953±0.008 |
| 4 | $V_{d\ (l)}$ | 1.0 | 1.3 | 1.5 | 0.9 | 1.2 | 1.7 | 1.266±0.145 |
| 5 | $T_{max\ (h)}$ | 1.2 | 1.4 | 1.6 | 1.1 | 1.3 | 1.8 | 1.02±0.115 |
| 6 | $C_{max}$(µg/ml) | 10.0 | 10.2 | 10.4 | 9.9 | 10.1 | 10.6 | 10.2±0.115 |
| 7 | $AUC_{0-12}$(µg/mlh) | 70.81 | 72.03 | 71.42 | 70.80 | 72.02 | 71.44 | 71.42±0.352 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 77.90 | 86.77 | 89.10 | 77.89 | 86.76 | 89.12 | 89.54±2.593 |

**Table 65 Group- V- Rifampicin nanoparticles (n=6, mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **6** | |
| 1 | $K_e$(1/h) | 0.421 | 0.435 | 0.438 | 0.420 | 0.434 | 0.440 | 0.431±0.008 |
| 2 | $K_a$ | 0.456 | 0.452 | 0.445 | 0.455 | 0.451 | 0.447 | 0.451±0.003 |
| 3 | $t_{1/2(h)}$ | 1.2 | 1.4 | 1.7 | 1.1 | 1.3 | 1.9 | 1.43±0.063 |
| 4 | $V_{d\ (l)}$ | 1.97 | 1.93 | 2.04 | 1.96 | 1.92 | 2.06 | 1.98±0.0031 |
| 5 | $T_{max\ (h)}$ | 1.8 | 1.9 | 1.4 | 1.7 | 1.8 | 1.6 | 1.7±0.07 |
| 6 | $C_{max}$(µg/ml) | 6.19 | 6.45 | 6.34 | 6.18 | 6.44 | 6.36 | 6.32±0.017 |
| 7 | $AUC_{0-12}$(µg/mlh) | 63.18 | 65.06 | 62.85 | 63.17 | 65.05 | 62.87 | 63.69±1.42 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 68.45 | 69.38 | 68.56 | 68.44 | 69.37 | 68.58 | 68.79±0.95 |

**Table 66 Group VI- Rifampicin nanoparticles +Isoniazid (n=6, mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| 1 | $K_e$(1/h) | 0.319 | 0.299 | 0.310 | 0.318 | 0.298 | 0.312 | 0.309±0.01 |
| 2 | $K_a$ | 0.276 | 0.318 | 0.321 | 0.275 | 0.317 | 0.323 | 0.305±0.63 |
| 3 | $t_{1/2(h)}$ | 2.1 | 2.3 | 2.5 | 2.0 | 2.2 | 2.7 | 2.3±0.04 |
| 4 | $V_{d\ (l)}$ | 0.99 | 0.97 | 0.99 | 0.98 | 0.96 | 1.0 | 0.98±0.13 |
| 5 | $t_{max\ (h)}$ | 1.61 | 1.63 | 1.68 | 1.60 | 1.62 | 1.70 | 1.64±0.63 |
| 6 | $C_{max}$(µg/ml) | 5.45 | 5.78 | 5.43 | 5.44 | 5.77 | 5.45 | 5.55±0.54 |
| 7 | $AUC_{0-12}$(µg/mlh) | 53.38 | 55.13 | 52.79 | 53.37 | 55.12 | 52.81 | 53.76±1.481 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 57.72 | 58.76 | 58.56 | 57.71 | 58.75 | 58.58 | 58.34±0.904 |

**Table 67 Group VII- Rifampicin -ascorbic acid nanoparticles (n=6, mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| 1 | $K_e$(1/h) | 0.493 | 0.498 | 0.493 | 0.492 | 0.497 | 0.495 | 0.494±0.01 |
| 2 | $K_a$ | 0.589 | 0.587 | 0.576 | 0.588 | 0.584 | 0.580 | 0.584±0.05 |
| 3 | $t_{1/2(h)}$ | 0.85 | 0.87 | 0.87 | 0.84 | 0.86 | 0.89 | 0.863±0.13 |
| 4 | $V_{d\ (l)}$ | 0.78 | 0.76 | 0.80 | 0.77 | 0.75 | 0.82 | 0.78±0.04 |
| 5 | $t_{max\ (h)}$ | 2.1 | 2.1 | 2.3 | 2.0 | 1.9 | 2.6 | 2.16±0.13 |
| 6 | $C_{max}$(µg/ml) | 10.78 | 9.78 | 9.79 | 10.77 | 9.77 | 9.81 | 10.11±1.33 |
| 7 | $AUC_{0-12}$(µg/mlh) | 71.04 | 69.89 | 70.13 | 71.03 | 69.88 | 70.15 | 70.35±1.368 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 79.89 | 80.12 | 76.77 | 79.88 | 80.11 | 76.79 | 79.92±2.031 |

**Table 68 Group -VIII- Rifampicin - ascorbic acid nanoparticles +isoniazid**

**(n=6, Mean±SD)**

| S.No | Parameters | Trails | | | | | | Mean±S.D |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| 1 | $K_e$(1/h) | 0.612 | 0.625 | 0.607 | 0.611 | 0.624 | 0.609 | 0.61±0.09 |
| 2 | $K_a$ | 0.534 | 0.530 | 0.531 | 0.533 | 0.528 | 0.534 | 0.531±0.12 |
| 3 | $t_{1/2(h)}$ | 0.91 | 0.98 | 0.96 | 0.90 | 0.97 | 0.98 | 0.95±0.013 |
| 4 | $V_{d\ (l)}$ | 1.0 | 1.3 | 1.5 | 0.9 | 1.2 | 1.7 | 1.266±0.63 |
| 5 | $T_{max\ (h)}$ | 1.28 | 1.40 | 1.60 | 1.27 | 1.30 | 1.80 | 1.42±0.26 |
| 6 | $C_{max}$(µg/ml) | 10.7 | 10.2 | 10.4 | 10.6 | 10.1 | 10.6 | 10.43±1.63 |
| 7 | $AUC_{0-12}$(µg/mlh) | 70.81 | 72.13 | 72.42 | 70.80 | 72.12 | 72.44 | 71.78±1.73 |
| 8 | $AUC_{0-\infty}$ (µg/mlh) | 77.90 | 82.78 | 81.17 | 77.89 | 82.77 | 81.19 | 80.61±2.18 |

## Cumulative data of pharmacokinetics study

### Table 69 (mean±S.D, n=6)

| Parameters | Rifampicin | Rifampicin + isoniazid | Rifampicin - ascorbic acid | Rifampicin - ascorbic acid +isoniazid | Rifampicin nanoparticle | Rifampicin nps+isoniazid | Rifampicin-ascorbic acid nps | Rifampicin - ascorbic acid nps+isoniazid |
|---|---|---|---|---|---|---|---|---|
| $K_e$(1/h) | 0.363±0.0184 | 0.301±0.004 | 0.424±0.004 | 0.518±.006 | 0.431±0.008 | 0.309±0.01 | 0.494±0.01 | 0.61±0.09 |
| $K_a$ | 0.425±0.0034 | 0.318±0.001 | 0.534±0.005 | 0.615±0.0005 | 0.451±0.003 | 0.305±0.63 | 0.584±0.05 | 0.531±0.12 |
| $T_{1/2(h)}$ | 1.823±0.064 | 2.533±0.088 | 1.594±0.008 | 0.953±0.008 | 1.43±0.063 | 2.3±0.04 | 0.863±0.13 | 0.95±0.013 |
| $V_{d\ (l)}$ | 0.93±0.011 | 0.863±0.017 | 1.603±0.068 | 1.266±0.145 | 1.98±0.0031 | 0.98±0.13 | 0.78±0.04 | 1.266±0.63 |
| $T_{max\ (h)}$ | 1.333±0.088 | 1.64±0.020 | 1.133±0.088 | 1.02±0.115 | 1.7±0.07 | 1.64±0.63 | 2.16±0.13 | 1.42±0.26 |
| $C_{max}$(µg/ml) | 5.906±0.052 | 4.086±0.046 | 9.276±0.020 | 10.2±0.115 | 6.32±0.017 | 5.55±0.54 | 10.11±1.33 | 10.43±1.63 |
| $AUC_{0-12}$(µg/mlh) | 33.96±1.411 | 27.81±0.877 | 69.146±2.906 | 71.42±0.352 | 63.69±1.42 | 53.76±1.481 | 70.35±1.368 | 71.78±1.73 |
| $AUC_{0-\infty}$(µg/mlh) | 61.05±0.798 | 53.53±1.775 | 82.48±2.099 | 89.54±2.593 | 68.79±0.95 | 58.34±0.904 | 9.92±2.031 | 80.61±2.18 |

**Table 70 Significance difference between groups (n=6, mean±SD)**

| Comparison | $K_e$ | $K_a$ | $t_{1/2}$ | $V_d$ | $T_{max}$ | $C_{max}$ | $AUC_{0-12}$ | $AUC_{0-\infty}$ |
|---|---|---|---|---|---|---|---|---|
| Group I VS Group II | *** | *** | *** | *** | *** | *** | *** | ** |
| Group I VS Group III | ** | ** | ** | ** | ** | ** | ** | *** |
| Group I VS Group IV | *** | * | *** | *** | *** | *** | * | *** |
| Group I VS Group V | *** | * | *** | *** | *** | *** | * | *** |
| Group I VS Group VI | *** | *** | * | *** | *** | *** | *** | * |
| Group I VS Group VII | *** | ** | *** | *** | *** | *** | ** | *** |
| Group I VS Group VIII | *** | *** | *** | *** | *** | *** | * | *** |
| Group II VS Group III | ** | *** | ** | ** | ** | ** | *** | ** |
| Group II VS Group IV | *** | ** | *** | * | *** | *** | ** | *** |
| Group II VS Group V | *** | *** | *** | *** | * | ** | *** | *** |
| Group II VS Group VI | *** | *** | *** | *** | *** | *** | *** | *** |
| Group II VS Group VII | *** | *** | *** | *** | *** | ** | *** | ** |
| Group II VS Group VIII | * | *** | * | ** | * | * | *** | * |
| Group III VS Group VI | ** | * | ** | ** | ** | ** | * | ** |
| Group III VS Group V | ** | ** | ** | *** | ** | ** | ** | ** |
| Group III VS Group VI | *** | ** | *** | *** | ** | ** | ** | *** |
| Group III VS Group VII | *** | *** | ** | *** | *** | ** | *** | ** |
| Group III VS Group VIII | *** | *** | *** | *** | *** | *** | *** | ** |
| Group IV VS Group V | *** | *** | *** | * | *** | *** | *** | *** |
| Group IV VS Group VI | * | *** | * | ** | * | * | *** | * |
| Group IV VS Group VII | ** | * | ** | ** | ** | ** | * | ** |
| Group IV VS Group VIII | ** | ** | ** | ** | ** | ** | ** | ** |
| Group V VS Group VI | ** | ** | * | *** | ** | ** | ** | * |
| Group V VS Group VII | *** | ** | *** | *** | *** | ** | ** | *** |
| Group V VS Group VIII | *** | *** | *** | *** | *** | *** | *** | ** |

***P<0.001, **P<0.01, *P<0.05

**Graphical representation of pharmacokinetics parameters**

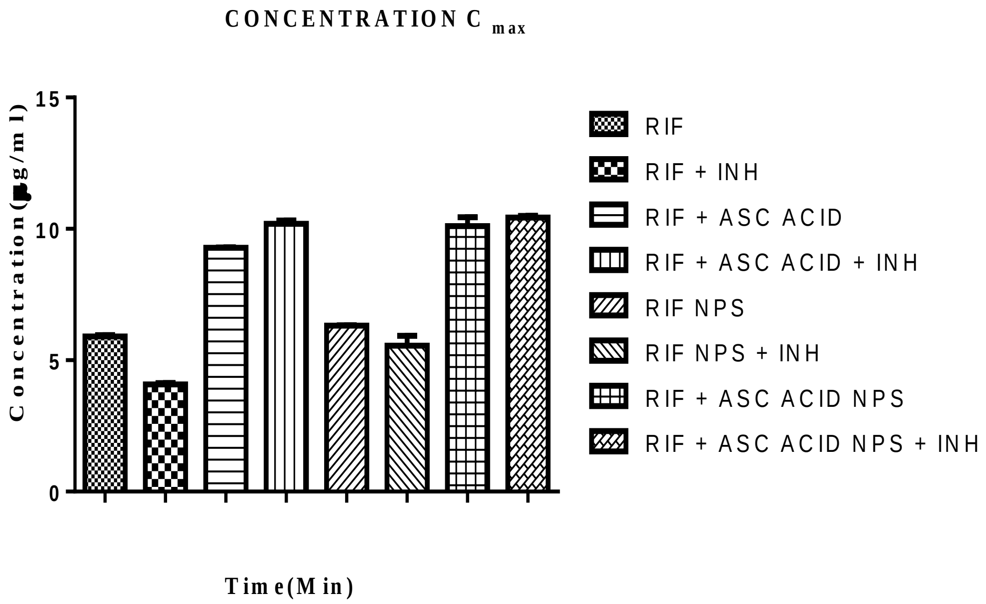


**Figure 68. C $_{max}$**

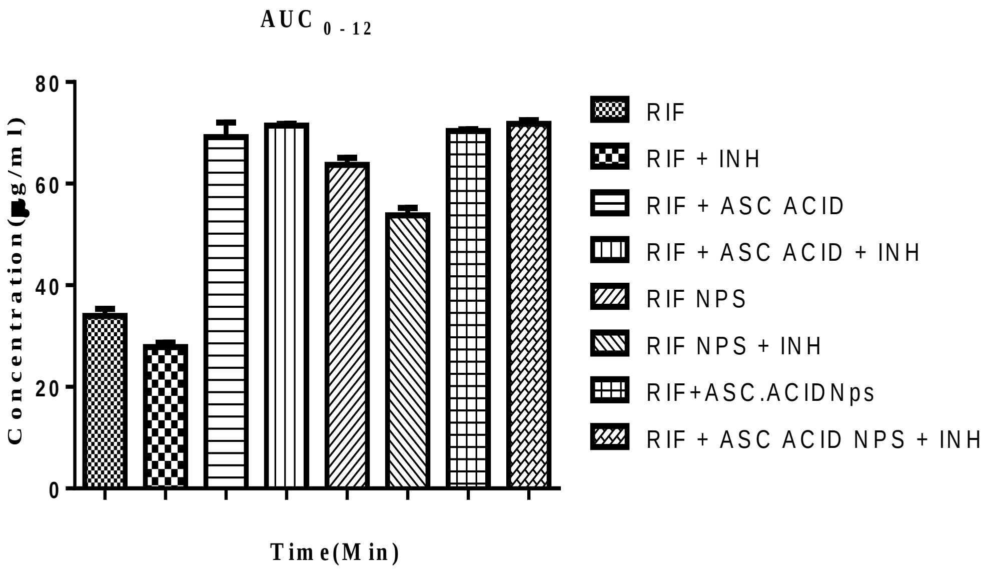


**Figure 69. AUC $_{0-12}$**

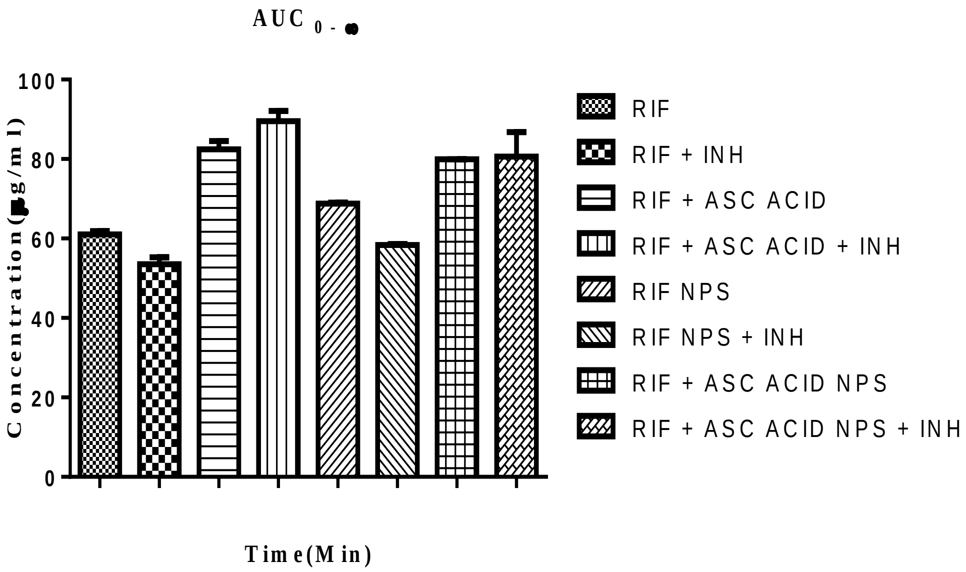


**Figure 70. AUC $_{0-\infty}$**

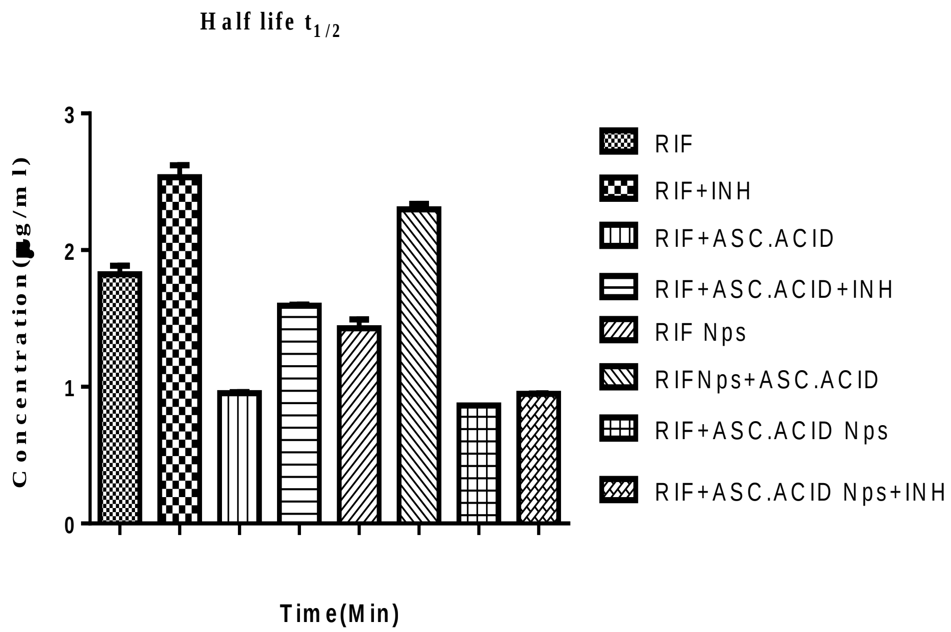


**Figure 71. Half life t $_{1/2}$**

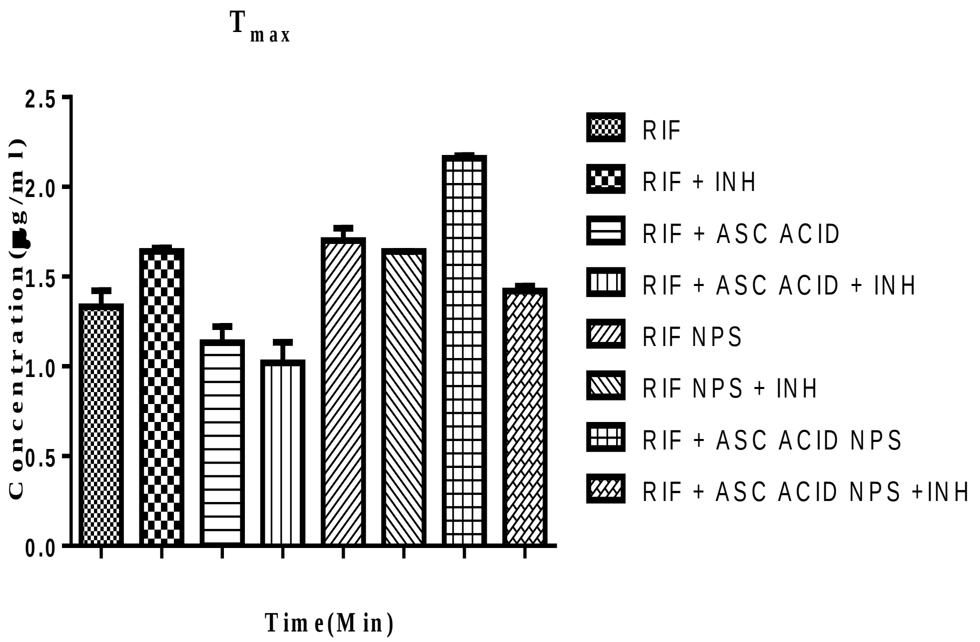


**Figure 72. $T_{max}$**

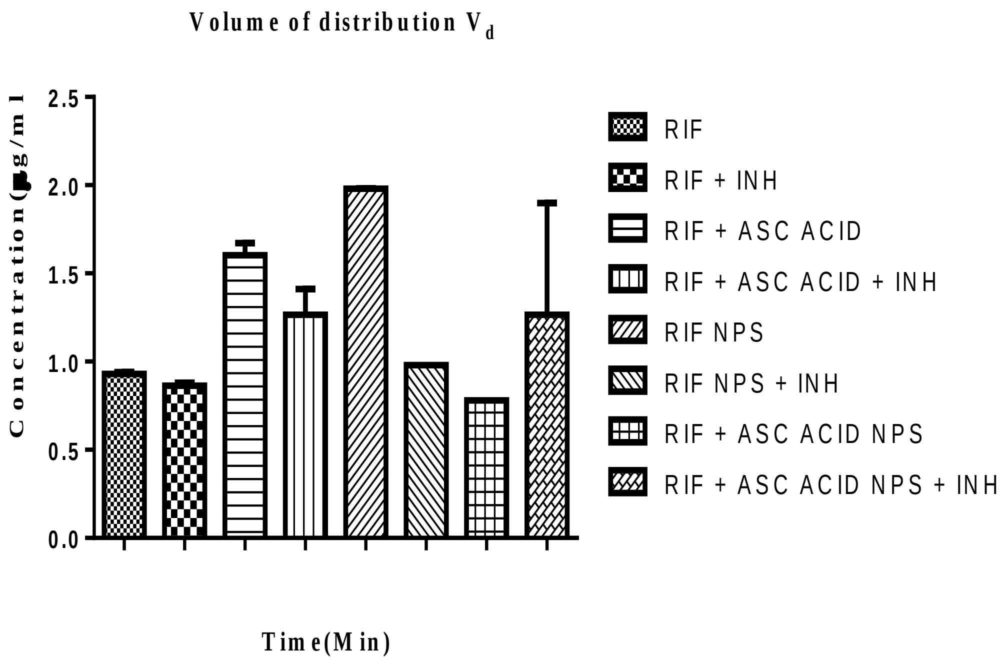


**Figure 73. Volume of distribution $V_d$**

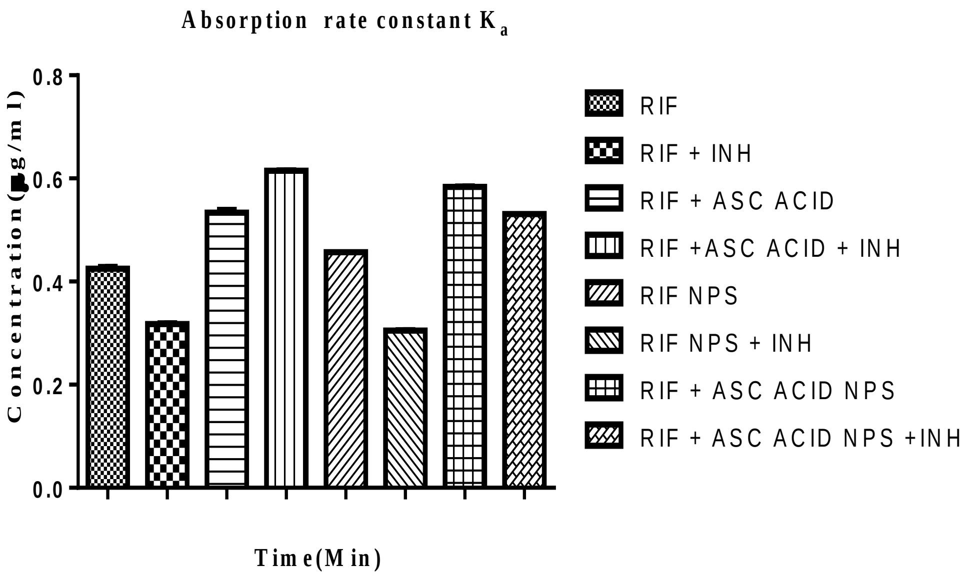


**Figure 74. Absorption rate constant $K_a$**

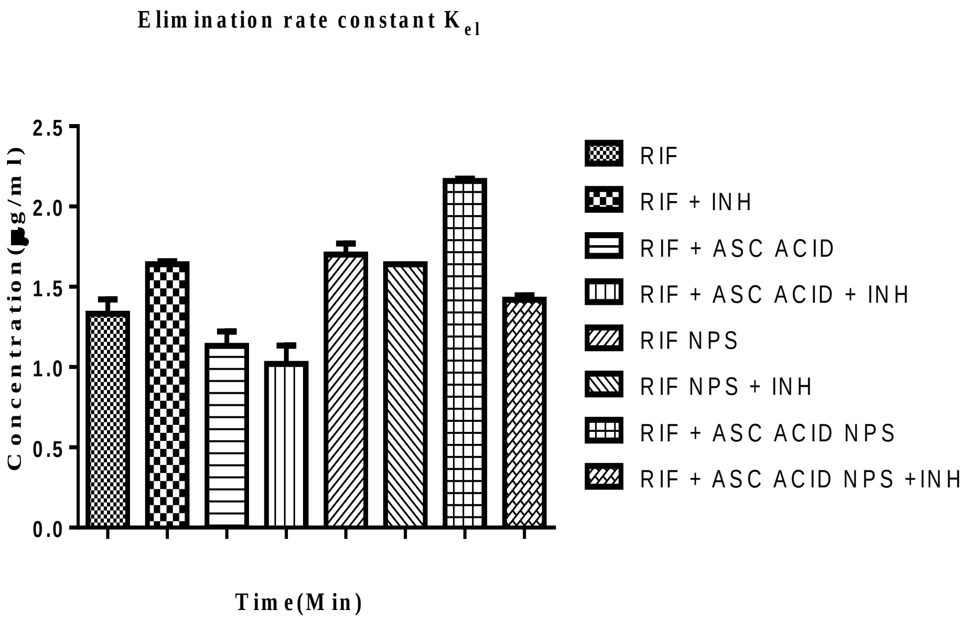


**Figure 75. Elimination rate constant $K_{el}$**

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INSTITUTIONAL ANIMAL ETHICAL COMMITTEE

(889/ac/05/CPCSEA/dated 29th April 2005)

## CERTIFICATE

This is certify that the project title **"Design and characterization of nanoparticulate oral delivery of anti-tubercular drugs"** has been approved by the IAEC as follows:

1. Date of Submission of Protocol: 22-01-11
2. Date of Approval: 27-01-11
3. Expiry Date: 30-09-11
4. Animals

| Species | Total Number of Animals Sanctioned |
|---|---|
| Rabbits | Male/female-36 |

5. Proposal number SVCP/IAEC/Ph.D./02/2011

27/01/11
Signature with Date:
(Prof. Dr. N. N. Rajendren)
**Chairman IAEC**

27/01/11
Signature with Date:
(Prof. Dr. E. P. Kumar)
**CPCSEA nominee**

# Acceptance: Ref. No: RJPBCS/2010-2626.

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Editor Comments: Minor revision

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